IMPACT D'UN RÉGIME RICHE EN ACIDE GRAS INSATURÉ SUR LES MÉTABOLITES SANGUINS AINSI QUE SUR LES MÉTABOLITES DU LAIT ET LA RELATION AVEC LES MICRO-ARN ET ARN MESSAGERS CO-EXPRIMÉS DANS LES GLANDES MAMMAIRES DE LA VACHE DE TYPE HOLSTEIN.

par

Adolf Ammah

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IMPACT OF DIETS RICH IN UNSATURATED FATTY ACIDS ON BLOOD AND MILK METABOLITES AND RELATIONSHIP WITH MICRO RNA AND MICRO RNA-MESSENGER RNA CO-EXPRESSION PATTERNS IN HOLSTEIN COW MAMMARY GLAND

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Adolf Ammah

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le jury a accepté la thèse de Monsieur Adolf Ammah dans sa version finale.

Membres du jury

Professeure Eveline Ibeagha-Awemu
Directrice de recherche
Chercheur Scientifique d'Agriculture et Agroalimentaire Canada

Professeur Nicolas Gévry
Co-directeur de recherche
Département de biologie

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Évaluateur externe
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Professeure Nathalie Bissonnette
Évaluateur interne
Chercheur Scientifique d'Agriculture et Agroalimentaire Canada

Professeur François Malouin
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Département de biologie

“The saddest aspect of life right now is that science gathers knowledge faster than society gathers wisdom.”

— Isaac Asimov

SOMMAIRE

Les mécanismes moléculaires et les processus biologiques qui surviennent dans la cellule suite à une diète comprenant un supplément d'acides gras insaturés (AGI), résultant à l'expression de gènes ou un ensemble de gènes coexprimé et éventuellement dirigés vers l'expression de phénotypes (métabolites sanguins et laitiers) ne sont pas encore bien compris. Considérant l'importance des AGI pour l'être humain, différentes techniques sont utilisées, alimentaire et génétique, afin d'augmenter le ratio d'acides gras bénéfiques dans le lait. Un supplément d'huile de lin (LSO) et huile de carthame (SFO) ont été utilisé, mais les effets résiduels de ces nutriments sur la physiologie de l'animal au niveau des métabolites sanguins et sur les composantes laitières ainsi que la durée des effets après le régime alimentaire. Dans la première partie de mon doctorat (chapitre 1), j'ai présenté des informations générales sur le lait et les composantes hématologiques bovines, la classification et l'importance des acides gras, le profilage du transcriptome des glandes mammaires etc. Dans la seconde partie de mon doctorat, chapitre 2, j'ai étudié les effets des suppléments alimentaires dans l'alimentation de la vache, et plus spécifiquement, la diète de la vache avec 5% de LSO ou 5% de SFO sur la composition du lait et métabolites sanguins des vaches Holstein en lactation.

Dans le troisième chapitre, j'ai déterminé la relation entre le sang, les métabolites du lait et des modules de micro-ARN démontrant l'interaction entre les modules micro -ARN et des données de co-expression ARNm / micro-ARN de la glande mammaire des mêmes animaux. L'analyse du transcriptome de l'ARNm a identifié 1 006 (460 en hausse et 546 en baisse) et 199 (127 en hausse et 72 en baisse) gènes qui étaient significativement différent régulé par LSO et SFO respectivement. De plus l'analyse du transcriptome de miARN a détecté 14 et 22 miARN qui étaient, significativement différemment régulés par LSO et SFO, respectivement. Cependant, puisque des réseaux de gènes et des facteurs de régulation peuvent agir de concert pour influencer l'expression phénotypique des caractères, l'évaluation de l'expression génique sans tenir compte des facteurs qui régissent leurs activités peuvent ne pas expliquer adéquatement le mécanisme biologique sous-jacent. De plus, les miARN interagissent avec les ARNm (s) pour réguler leur expression et par conséquent les processus biologiques et il est donc important

d'étudier leurs effets synergiques sur l'expression phénotypique des caractères (sang et composants laitiers). Pour ces raisons, j'ai déterminé la relation entre les traits (lait et paramètres sanguins) et miRNA des paires de miARN et de miARN après une supplémentation alimentaire avec 5% de LSO ou 5% de SFO. Plus spécifiquement, dans la troisième partie de mes études de doctorat, j'ai étudié les modèles de co-expression des miARN à haute interaction et les paires miRNA-ARNm et le réseau de régulation clé des gènes qui influencent le phénotype du sang et du lait après supplémentation alimentaire avec les AGI.

Les modifications alimentaires représentent un moyen efficace pour modifier rapidement la composition des matières grasses du lait. Cependant, les effets de la supplémentation alimentaire, sont totalement révocables suite aux conditions d'alimentation changeantes couplées à une faible efficacité de conversion des aliments en AGI dans le lait en raison de la biohydrogénation ruminale des acides gras alimentaires dans le rumen de l'animal. De plus, des études de certains gènes ont démontré que les polymorphismes mononucléotidiques (SNP) dans certains gènes lipogéniques clés comme la diacylglycérol o-acyltransférase 1 (*DGAT1*), la régulation des stérols la protéine liant l'élément 1 (*SREBP1*) et la stéaroyl-CoA désaturase (*SCD*) influencent la composition du lait en graisse, ce qui explique également une partie de la variation génétique des indices d'insaturation des graisses du lait Holstein et vaches brunes. De ce fait, j'ai étudié les associations entre les SNPs sur les gènes qui ont été différentiellement exprimés à la suite d'une supplémentation alimentaire avec UFA avec des profils d'acides gras synthétisés de novo des vaches Holstein canadiennes comme quatrième partie de mes études de doctorat (chapitre 4). A cette fin, les SNPs dans les gènes qui ont été exprimés différemment suite d'une supplémentation alimentaire avec UFA ont été étudiés pour leur association avec le lait synthétisé de novo des acides gras dans le lait Holstein de 18 troupeaux au Québec.

En conclusion, cette étude fournit des informations sur les mécanismes moléculaires qui se produisent dans la glande mammaire bovine suite d'une supplémentation alimentaire avec 5% de LSO et 5% de SFO.

Mots clés: métabolites sanguins, composants du lait, polymorphisme à nucléotide simple, acide gras, miARN, co-expression miRNA-ARNm, association génétique, vache Holstein.

SUMMARY

The effect of unsaturated fatty acids (USFA) as dietary supplement on the expression of genes or set of co-expressed genes that eventually direct the expression of phenotypes (blood and milk metabolites) are not fully understood. Considering the importance of USFA for human, different techniques (dietary and genetics) have been used to increase the proportion of beneficial FA in milk. Dietary supplementation with LSO and SFO has been used but it is not clear what residual effects these nutrients have on the physiology of the animal, including blood metabolites and milk components, or for how long effects are active after withdrawal from the diet. In the first part of my Ph.D. study (Chapter 1), I presented general background information, notably on bovine milk, milk fat and its origin, mammary gland transcriptome profiling, naming, classification and importance of FA, factors that inlfunece milk fatty acid composition and blood components. In the second part (Chapter 2), I examined the treatment and post treatment effects of supplementation of cows' diet with 5% LSO or 5% SFO on milk composition and blood metabolites of lactating Holstein cows. Thirdly (Chapter 3), I determined the relationship between the blood and milk metabolites and high interacting microRNA (miRNA) modules and evaluated mRNA/miRNA co-expression from the mammary gland of the same animals. The mRNA transcriptome analysis identified 1,006 (460 up and 546 down-regulated) and 199 (127 up and 72 down-regulated) genes that were significantly differentially regulated by LSO and SFO, respectively. Meanwhile the miRNA transcriptome analysis detected 14 and 22 miRNAs that were significantly differentially regulated by LSO and SFO, respectively. Since a network of genes and regulatory factors work in concert to influence the phenotypic expression of traits, assessment of gene expression without considering the factors that regulate their activities may not adequately explain the complex biological mechanisms underlying the expression of milk components and blood metabolites. Moreover, miRNA interact with mRNA to regulate their expression and consequently biological processes, so it is important to study their synergistic effects on the phenotypic expression of traits (blood and milk components). Therefore, the relationship between these traits and miRNA modules and also the association of miRNA and mRNA following dietary supplementation with 5% LSO or 5% SFO were studied. In the fouth

part (Chapter 3) of my PhD study, I investigated the co-expression patterns of high interacting miRNAs and miRNA-mRNA pairs and the key regulatory network of genes that influence blood and milk phenotypes following dietary supplementation with USFA.

Dietary modifications through feeding trials is an effective way to rapidly modify milk fat composition. However, the effects of dietary supplementation are totally revocable when feeding conditions change, coupled with low conversion efficiency of dietary FA into milk USFA due to ruminal biohydrogenation of dietary FA in the rumen of the animal. Furthermore, candidate gene studies have demonstrated that SNP in some key lipogenic genes like diacylglycerol o-acyltransferase 1 (*DGAT1*), sterol regulatory element binding protein-1 (*SREBP1*) and stearoyl-CoA desaturase (SCD) influence milk fat composition, which also explains part of the genetic variation of milk fat unsaturation indices seen in Holsteins and Brown Swiss cows**.** Therefore, I investigated the associations between SNPs on genes that were differentially expressed as a result of dietary supplementation with USFA with *de novo* synthesized fatty acid profiles of Canadian Holstein cows as the fifth part of my Ph.D. study (Chapter 4). To achieve this, SNPs in genes that were differentially expressed as a result of dietary supplementation with USFA were studied for their association with milk *de novo* synthesized FA in milk from Holstein cows in 18 herds in Quebec.
In conclusion, this study provides insights on the molecular mechanisms that occur in the bovine mammary gland following dietary supplementation with 5% LSO and 5% SFO.

Key words: blood metabolites, milk components, single nucleotide polymorphism, fatty acid, miRNA, miRNA-mRNA co-expression, genetic association, Holstein cow.

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CONTENTS

List of Abbreviations

ACACA	Acetyl-CoA carboxylase alpha
AGPAT	1-acyl-glycerol-3-phosphate-acyltransferase
ALA	Alpha linolenic acid
BHBA	Beta hydroxybutyric acid
CD36	Cluster of differentiation 36
CLA	Conjugated linoleic acid
CP	Control period
DM	Dry matter
DNA	Deoxyribonucleic acid
FA	Fatty acids
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FAME	Fatty acid methyl esters
FAS	fatty acid synthase
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
KME	Module eigengene
LA	Linoleic acid
LCFA	Long chain fatty acids
LPL	lipoprotein lipase
LSO	Linseed oil
MFD	Milk fat depression
miRNA	micro Ribonucleic acid
mRNA	messenger ribonucleic acid
MUFA	Monounsaturated fatty acid
MUN	Milk urea nitrogen
NEFA	Non-esterified fatty acids
PTP	Post treatment period

PUFA	Polyunsaturated fatty acids
qPCR	Quantitative real time polymerase chain reaction
RNAs	Ribonucleic acid
RNA-seq	RNA sequencing
SCC	Somatic cell count
SCD1	Stearoyl-CoA desaturase 1
SCFA	Short chain fatty acids
SFA	Saturated fatty acids
SFO	Safflower oil
SNP	Single nucleotide polymorphisms
SREBP1	Sterol regulatory element binding protein 1
TAG	Triacylglycerides
TF	Transcription factors
TMR	Total mixed ration
TP	Treatment period
USFA	Unsaturated fatty acids
VLDL	Very low-density lipoprotein
WGCNA	Weighted gene co-expression network analysis

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CHAPTER 1

GENERAL INTRODUCTION

The introduction of this thesis provides a review of bovine milk and blood components, classification and importance of milk FA, factors affecting the composition of milk FA and blood metabolites, mammary gland transcript profiling, the effects of dietary unsaturated fatty acids (USFA) on blood and milk components, and the association of SNP with milk *de novo* synthesized FA. Studies on miRNA and miRNA-mRNA co-expression analysis were also reviewed. The results of the studies in this thesis are presented in the form of manuscripts in chapters 2, 3 and 4.

1.0 Bovine milk and blood components, classification and importance of fatty acids

1.1 Bovine milk

Bovine milk and its products have a long history in the nutrition of humans and are a rich source of proteins, energy, minerals (e.g. calcium), and vitamins (A, B, D, E and K) (Knutsen et al., 2018). Additionally, it contains hormones, growth factors, enzymes, polyamines, bioactive peptides (Haug et al., 2007) that are needed for the growth of the calf. Because of these properties, milk is said to be a complete food with true nutraceutical properties (Sommella et al., 2018). The composition of bovine milk is approximately 4.6% lactose, 87% water, 3.4% protein, 4.2% fat, 0.1% vitamins, 0.8% minerals (Mansson, 2001). However, this composition is not fixed as it undergoes continuous change depending on feeding strategies, breed, cow management, lactation stage and season (Mansson, 2001).

Blood on the other hand, transports nutrients and metabolites from various organs of the body like liver and intestines to the mammary gland where they are used for the synthesis of different components found in milk. Bovine milk also supplies fat that has some nutritional importance to man.

1.2 Bovine milk fat

Milk fat provides energy for the body and is an important determinant of milk nutritional quality. It consists mainly of triglycerides or triacylglycerols (TAG) which make up approximately 98% of milk fat (Hein et al., 2017; Jensen and Newburg, 1995). TAG is formed when a single molecule of glycerol combines with three FA (Figure 1.1). In addition to TAG, milk also contains 0.02% diacylglycerols and monoacylglycerols, while NEFA and phospholipids consist of 0.10–0.44% and 0.20–1.00% respectively (Jensen, 2002). Milk fat is by produced in the mammary epithelial cells and secreted as fat globules (MacGibbon and Taylor, 2006), which are mainly composed of globules of triaclyglyceride surrounded by lipid membrane.

Typical milk fat from dairy cows contains 70% SFA, 25% MUFA and 5% PUFA (Gottardo et al., 2017; Grummer, 1991). Considering the impact of FA on human health (Hu et al., 1999; Parodi, 1999; Simopoulos, 2003), milk fat composition with potential positive effects on human health should contain 60% MUFA, 30% SFA and 10% PUFA (Pascal, 1996; Soyeurt et al., 2006).

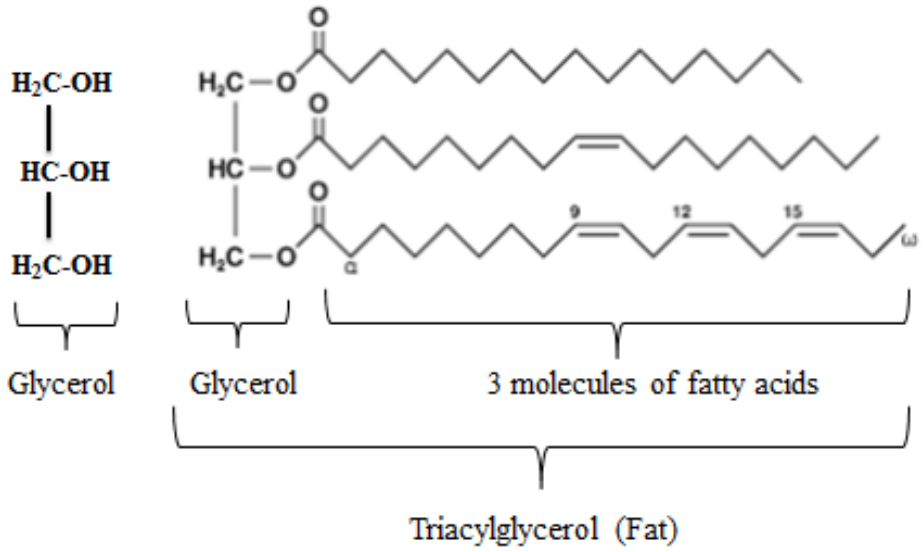

Figure 1.1 A triacylglycerol molecule made up of three fatty acids held together through an ester linkage with glycerol.

1.3 Origin and nature of bovine milk fatty acids

The FA in milk are *de novo* synthesized in the mammary glands (Knutsen et al., 2018; Bauman and Griinari, 2003), or taken up (preformed FA) from blood circulation (Bauman and Griinari, 2003). Circulating acetate and 3-hydroxybutyrate are used as substrates for *de novo FA* synthesis. These precursors are derived from the fermentation of hemicellulose and cellulose as a result of microbial processes in the rumen (Jensen 2002). The cellulose and associated materials are changed to hydroxybutyrate, propionate, acetate, which enter the circulation and butyrate is changed to hydroxybutyrate in the ruminal wall (Jensen 2002). *de novo* synthesized FA accounts for 95%

of all C4:0 to C14:0 and about 50% of C16:0 secreted in milk (Shingfield et al., 2010). Fatty acids are building blocks of phospholipids and glycolipids in biological membranes. The body cells use either glucose or FA as energy source.

Preformed FA in the intestines move through the intestinal walls and are transported as lipoproteins to the mammary gland. All FA with 18 carbons or longer chain are derived from circulating plasma lipids (Chilliard et al., 2000). To facilitate entry of the preformed FA into the mammary gland, TAG which is the main component of the lipoprotein is hydrolysed to FA (Figure 1.2) (Bernard et al., 2008; Shingfield et al., 2010). This hydrolysis by lipoprotein lipase results in the formation of low density lipoprotein and very low-density lipoprotein (VLDL) or chylomicrons and this supplies the preformed FA found in milk (Figure 1.2). The major preformed FA in bovine milk are C16:0, C18:0 and C18:1 (Mansbridge and Blake, 1997).

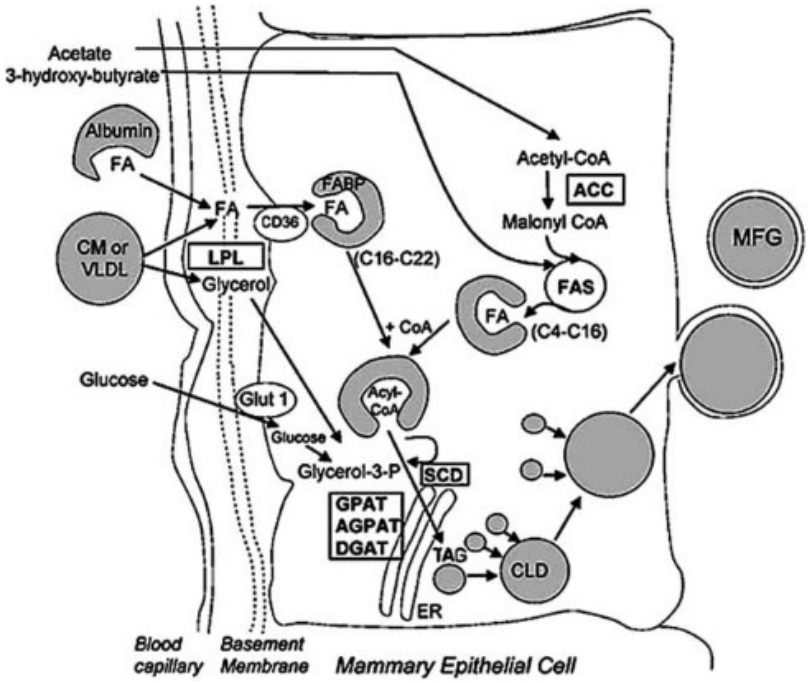

Figure 1.2 Uptake of preformed FA from the blood circulation and milk fat synthesis by bovine mammary epithelial cells. Acetate and 3-hydroxy butyrate are taken up by the mammary cells. Acetate eventually form acetyl CoA, while through the action of *ACCA* and

FAS, Acetate and 3-hydroxy butyrate eventually produces TAG which is excreted as milk fat globule. Uptake of preformed FA (albumin bound FA and CM or VLDL) from circulation by the action of *FABP*, *CD36* and *LPL* to eventually form TAG which is again excreted as MFG. Abbreviations: TAG (triacylglyerol), ER (endoplasmic reticulum), FA (fatty acid), CM (chylomicron), MFG (milk fat globule), VLDL (very low density-lipoprotein), CLD (cytoplasmic lipid droplet), *CD36* (Cluster of differentiation 36), *Glut 1* (glucose transporter 1). Some genes involved in TAG synthesis are *AGPAT* (1-acyl glycerol 3-phospate acyl transferase), *GPAT* (glycerol-3 phosphate acyl transferase), and *DGAT 1* (diacylglycerol acyltransferase 1). Fatty acid desaturation: *SCD* (Stearoyl-CoA desaturase). Fatty acid uptake: *LPL* (lipoprotein lipase). For intracellular transport: *FABP* (fatty acid binding protein) and *de novo* FA synthesis: *ACC* (acetyl-CoA carboxylase); *FAS* (fatty acid synthase). Adapted, from Bernard et al., (2008).

Preformed fatty acids that eventually reach the mammary epithelial cells undergo further modifications through extensive desaturation of medium and long chain SFAs by stearoyl-CoA desaturase 1 (*SCD1*) also known as delta 9 desaturase (Shingfield et al., 2010). Furthermore, the activities of delta 5 or *FADS1* and delta 6 or *FADS2* also results in formation of long chain PUFAs like docosapentaenoic acid and eicosapentaenoic acid with beneficial health effects (Lee et al., 2016). Polyunsaturated fatty acids tend to be concentrated in the phospholipids and cholesterol esters of the high-density lipoprotein and the uptake of FA from high density lipoprotein by the mammary gland is very poor which may partially explain low levels of PUFA in bovine milk (Mansbridge and Blake, 1997).

1.4 Naming and classification of fatty acids

Fatty acids are compounds that contain a long hydrocarbon chain with a terminal carboxylic acid group. The elements that make up FA are carbon (C), hydrogen (H) and oxygen (O) arranged as a carbon skeleton with a carboxyl group (-COOH) at the end. Fatty acid nomenclature is based on the systematic nomenclature recommended by the International Union of Pure and Applied Chemistry (IUPAC-IUB Commission on Nomenclature, 1978). Based on

this system, FA are named according to the number of carbon atoms, the number and position of double bonds relative to the carboxyl group and also the configuration of the double bonds.

According to carbon number, FA can either be SCFA (< 6 carbon atoms, for example butyric acid or butanoic acid [C4:0], medium chain (6 to 12 carbon atoms, for example octanoic acid or caprylic acid [C8:0], LCFA (12 to 20 carbon atoms, for example stearic acid or octadecanoic acid [C18:0] or very long chain (>20 carbon atoms, for example docosapentaenoic acid [C22:5n3]). The names and chemical structure of some FA commonly found in bovine milk are shown in Table 1.1.

According to degree of saturation, FA that contain the maximum number of hydrogen atoms that their molecular structure can permit are known as SFA. They are characterized by the absence of double bonds between carbon atoms. Saturated fatty acids are the most stable of all classes of FA found in bovine milk and have relatively high melting points.

Table 1.1 Common name and chemical structure of fatty acids commonly found in bovine milk.

Fatty acid	Common name	Chemical structure
Saturated fatty acids		
C4:0	Butyric acid	$CH_3(CH_2)_2COOH$
C6:0	Caproic acid	$CH_3(CH_2)_4COOH$
C8:0	Caprylic acid	$CH_3(CH_2)_6COOH$
C10:0	Caprinic acid	$CH_3(CH_2)_8COOH$
C11:0	Undecanoic acid	$CH_3(CH_2)_9COOH$
C12:0	Lauric acid	$CH_3(CH_2)_{10}COOH$
C13:0	Tridecylic	$CH_3(CH_2)_{11}COOH$
C14:0	Myristic acid	$CH_3(CH_2)_{12}COOH$
C15:0	Pentadecylic	$CH_3(CH_2)_{13}COOH$
C16:0	Palmitic acid	$CH_3(CH_2)_{14}COOH$
C17:0	Margaric acid	$CH_3(CH_2)_{14}COOH$
C18:0	Stearic acid	$CH_3(CH_2)_{16}COOH$
C20:0	Arachidonic acid	$CH_3(CH_2)_{18}COOH$
C22:0	Docosanoic acid	$CH_3(CH_2)_{20}COOH$

C23:0	Tricosanoic acid	$CH_3(CH_2)_{21}COOH$
C24:0	Lignoceric acid	$CH_3(CH_2)_{22}COOH$
C26:0	Cerotic acid	$CH_3(CH_2)_{24}COOH$
Monounsaturated fatty acids		
C14:1, *trans*-9	Myristelaidic acid	$CH_3(CH2)_3(CH=CHCH2)(CH2)_6COOH$
C14:1, *cis*-9	Myristoleic acid	$CH_3(CH2)_3(CH=CHCH2)(CH2)_6COOH$
C16:1, *trans*-9	Palmitelaidic acid	$CH_3(CH2)_7(CH=CHCH2)(CH2)_4COOH$
C16 :1, *cis*-9	Palmitoleic acid	$CH_3(CH2)_7(CH=CHCH2)(CH2)_4COOH$
C18:1, *trans*-9	Elaidic acid	$CH_3(CH2)_7(CH=CHCH2)(CH2)_7COOH$
C18:1, *cis*-9	Oleic acid	$CH_3(CH2)_7(CH=CHCH2)(CH2)_7COOH$
C18:1, *trans* 11	*trans* vaccenic acid	$CH_3(CH_2)_5(CH=CHCH_2)(CH_2)_8COOH$
Polyunsaturated fatty acid		
C18:2, *cis*-9 *trans*-11	*c*is-9, *trans*-11 CLA	$CH_3(CH_2)_5(CH=CH)_2(CH_2)_7COOH$
C18:2, *trans*-10, *cis*-12	*trans*-10, *cis*-12 CLA	$CH_3(CH_2)_4(CH=CH)_2(CH_2)_8COOH$
C18:2, *trans*-9,12	*trans*-linoleic acid	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$
C18:2, *cis*-9-12	Linoleic acid	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$
C18: 3, *cis*-9,12,15	Alpha linolenic acid (omega 3)	$CH_3(CH_2)(CH=CHCH_2)_3(CH_2)_6COOH$
C20:3, *cis*- 8,11,14	Dihomogamma linolenic acid (omega 6)	$CH_3(CH_2)_4(CH=CHCH_2)_3(CH_2)_5COOH$
C20:4, *cis*-5,8,11,14	Arachidonic acid (omega 6)	$CH_3(CH_2)_3(CH=CHCH_2)_4(CH2)_3COOH$
C20:5, *cis*- 5,8,11,14,17	Eicosapentanoic acid (omega 3)	$CH_3(CH_2)(CH=CHCH_2)_5(CH_2)_2COOH$
C20: *cis* 6, 8, 10, 12,14	Bosseopentaenoic acid (omega 6)	$CH_3(CH_2)(CH=CHCH_2)_5(CH_2)_2COOH$
C22:5, *cis*-7,10,13,16,19	Docosapentaenoic acid (omega 3)	$CH_3(CH_2)(CH=CHCH_2)_5(CH_2)_4COOH$

On average, bovine milk fat contains about 7% short chain SFA (C4:0 to C8:0), 15-20% medium chain SFA (C10:0 to C14:0) and 42-48% long chain SFA (C16:0 and higher).

Unsaturated fatty acids are characterized by the presence of one (MUFA) or more (PUFA) double bonds in their molecular structure. They (Figure 1.3 A and B, and Table 1.1) generally have a lower melting point than their saturated counterparts. Long chain PUFAs (20 carbon atoms or more) are classified into three principal families, omega 6 (n-6 or ω-6), omega 3 (n-3 or ω-3) and omega 9 (n-9 or ω-9) families, according to the position of the terminal double bond (Glaser et al., 2010). The main omega 3 and omega 6 families are linoleic acid (LA) and alpha linolenic acid (ALA). Linoleic acid and ALA cannot be synthesised in mammals, so they must be provided by the diet and are therefore defined as essential FA (Glaser et al., 2010). Most of the USFA in bovine milk are either from dietary sources or synthesized in the mammary gland.

They are formed from their saturated counterparts through the activities of the enzyme *SCD1* or through further desaturation of LA and ALA by the activities of *FADS1* and *FADS2*. Unsaturated FA which are isomers of LA with conjugated double bonds are collectively called CLA. Depending on the position and geometry of the double bonds, several isomers of CLA have been identified (Nirvair et al., 2007) including major forms like *cis* 9, *trans* 11-CLA and *trans* 10, *cis* 12-CLA, and minor forms like *trans* 7, *trans* 9-CLA; *cis* 9, *cis* 11-CLA; *trans* 9, *trans* 11-CLA. Unsaturated FA are further categorized into *cis* (Figure 3C and E) and *trans* (Figure 1.3D and H) isomers depending on the orientation of the hydrogen atoms in respect of the double bonds. A *cis* configuration means that adjacent hydrogen atoms are on the same side of the double bond. *Trans* configuration (across or on opposite side) by contrast means that, the next two hydrogen atoms are bound to opposite sides of the double bond. In the *trans* configuration, there is no bend in the carbon chain and their shapes are similar to SFA (Figure 1.3H). In the *cis* isomers, the rigidity of the double bond halts its conformation and causes the chain to bend and restricts the conformational freedom of the FA (Figure 1.3E). The more double bonds the chain has in the *cis* configuration, the less flexible it becomes. When a chain has many *cis* bonds; it becomes quite curved in its most accessible conformations. For example, oleic acid with one double bond has a "kink" shape (Figure 1.3E), whereas LA with two double bonds has a more pronounced bend (Figure 1.3F) and ALA with three double bonds is almost curved like a letter C (Figure 1.3G).

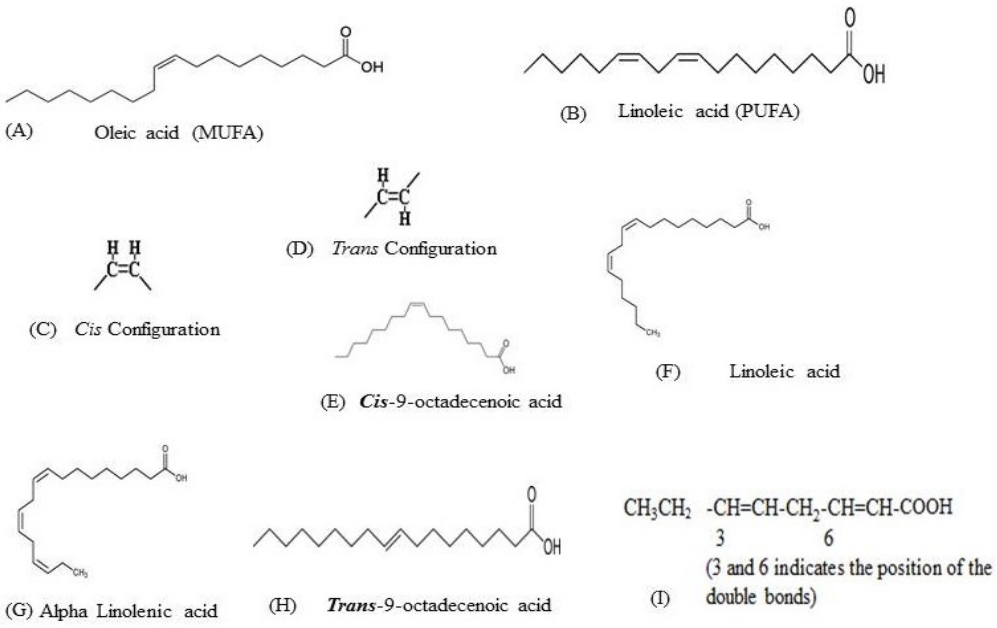

Figure 1.3 Some common fatty acids in bovine milk showing *cis*, *trans* positions and their effect on the structure of fatty acids and also the degree of saturation.

The 'telegraphic' system of nomenclature indicates the carbon, the number of carbon atoms and also the number of double bonds (Figure 1.4A). Based on this type of classification we can name the SCFA, C4:0 as butyric acid or butanoic acid. Letter 'C' stands for carbon; '4' for number of carbon atoms and '0' for the number of double bonds.

Naming of USFA using the telegraphic system can start either from the carboxyl or methyl end of the chain. When numbering from the carboxyl end, carboxyl C is number 1 as shown in Figure 1.4B.

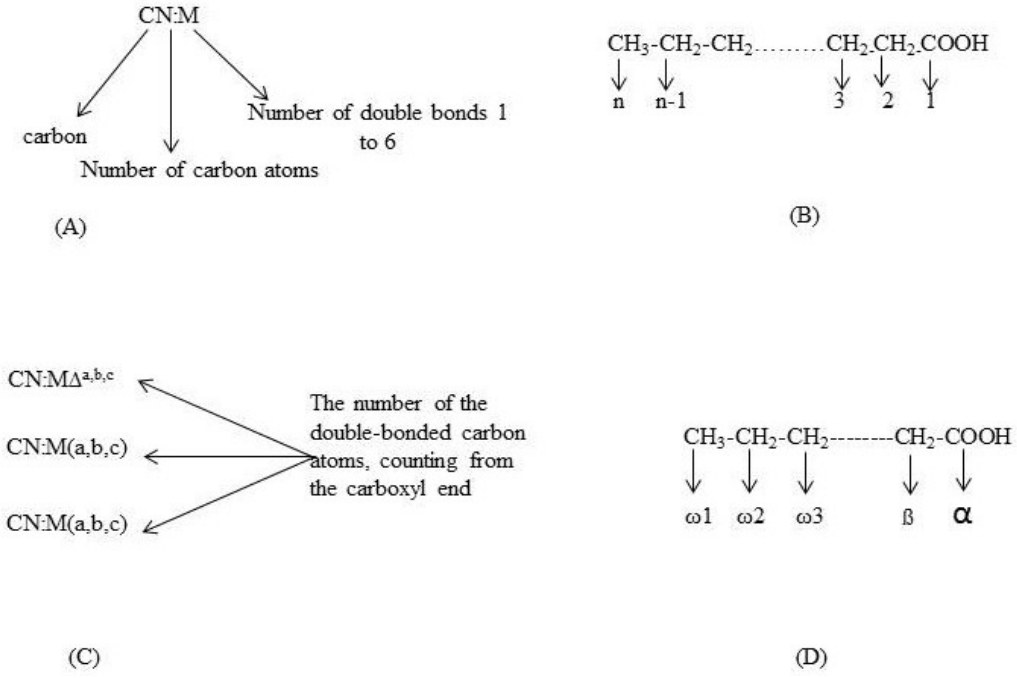

Figure 1.4 Telegraphic system of nomenclature of fatty acids

When numbering from the methyl end- methyl C is marked ω1 (omega-1) or n-1 (Figure 1.4B and 1.4D). The following USFA can be named using the above systems as follows: C18:1, *cis*-9 = oleic acid or *cis*-9-octadecanoic acid and C18:1, *trans*-9 = elaidic acid or *trans*-9-octadeconoic acid.

1.5 Importance of fatty acids

Milk fat is a major source of energy for the body and also an important determinant of milk nutritional quality and organoleptic properties. Dietary fats provide the medium for the absorption of fat-soluble vitamins; they are a primary contributor to the palatability of food and are crucial to the proper development and survival during the early stages of life (embryonic development) and early growth after birth on to infancy and childhood. Milk supplies USFA with positive effects on human health (Parodi, 2005; Salter et al., 2013). Unsaturated FA are

associated with decreased risk of cardiovascular diseases (stroke, high blood pressure, heart failure and coronary heart diseases), inflammatory diseases and some types of cancers (Kris-Etherton and Innis, 2007; Griel and Kris-Etherton, 2006; Parodi, 2005). Unsaturated FA also play a protective role against several chronic diseases and therefore, may increase human longevity (Solfrizzi et al., 2005). Long chain omega-3 FA play a key role as structural components for the development of the brain and the central nervous system (Dyall, 2015). In restricted environments such as when FA are part of a phospholipid in a lipid bilayer, or triglycerides in lipid droplets, *cis* bonds limit the ability of FA to be closely packed, and therefore could affect the melting temperature of the membrane or of the fat. Intake of the PUFAs, eicosapentaenoic acid and docosahexaenoic acid have demonstrated physiological benefits on blood pressure, heart rate, triglycerides, inflammation, endothelial function, cardiac diastolic function, and consistent evidence for a reduced risk of fatal coronary heart disease and sudden cardiac death (Tavazzi et al., 2008; Mozaffarian and Rimm, 2006; Yokoyama et al., 2007; Wang et al., 2006; Marik and Varon, 2009). Docosahexaenoic acid also plays a major role in development of the brain and retina during foetal development and the first two years of life (Cetin and Koletzko, 2008; Decsi and Koletzko, 2005; Helland et al., 2008). Some SFAs in milk have cholesterol-raising effects, mainly lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0). However, the SFA C18:0 has been reported to be as effective as C18:1 in reducing plasma cholesterol levels (Bonanome and Grundy, 1988). In addition, butyric acid (C4:0) is known to modulate gene function and may play a role in cancer prevention (colon cancer mainly by preventing cell proliferation and inducing apoptosis) (Kiefer et al., 2006, Hu et al. 2011). The butyrate is from the result of anaerobic bacteria fermentation of carbohydrate in the colon (Siregara et al., 2016). Additionally, C8:0 and C10:0 may have antiviral activities (Hilmarsson et al., 2006; Thormar, and Hilmarsson, 2007) and C8:0 in particular has been reported to delay tumor growth (German, 1999). To improve wellness, a reduction in the overall consumption of SFA, trans FA and cholesterol has been put forth while emphasis has been placed on the need to increase intake of USFA (Kris-Etherton and Innis, 2007; Salter et al., 2013).

1.6 Factors that influence milk fatty acid composition

Fat is the most variable component of milk (40 ± 16 g/l (Jensen et al., 1995), having a coefficient of variation of 40% compared to protein (9.2 ± 1.8 g/l (Atkinson and Lonnerdal, 1995), with a coefficient of variation of 19% and lactose (63 ± 2 g/l (Newburg et al., 1995), with a coefficient of variation of 3.1%. Furthermore, its composition is affected by several factors including genetics, breed, stage of lactation, parity, environment (e.g. nutrition), season and epigenetics.

1.6.1 Stage of lactation

The lactation cycle is divided into three stages which are, early lactation (less than 100 days in milk), mid lactation (100 to 200 days in milk) and late lactation (more than 200 days in milk). Depending on the fat source (*de novo* synthesised or preformed FA) the changes in milk fat composition during lactation may follow different patterns. As lactation progresses, the relative proportions of most *de novo* FA (short and medium chain FA) increases, whereas proportions of most preformed fatty acids (long chain FA) decreases (Palmquist et al., 1993; Secchiari et al., 2003; Kay et al., 2005; Garnsworthy et al., 2006; Kgwatalala et al., 2009). During early lactation, cows are usually in negative energy balance. Negative energy balance is associated with an increase in milk C16:0 and C18:0, which suggests mobilization of body fat reserves (Stoop et al., 2009) and incorporation of LCFAs, especially C18:1 into milk fat (Belyea and Adams, 1990). This incorporation of LCFAs especially C18:1 into milk fat inhibits *de novo* SCFA synthesis and may account for the lower proportions of short and medium chain FA at the early stage of lactation (Bauman and Griinari, 2003). Afterwards, there is a consistent increase in the proportion of short and medium chain FA with increasing lactation accompanied by compensatory decrease in the proportion of all LCFAs like C18, with 90% of C6 to C16 FA production occurring at around 8 weeks in lactation (Palmquist et al., 1993).

1.6.2 Parity

Though there is limited data in literature on the effect of parity on fatty acid composition in milk are limited, there is no doubt that this factor affects milk fatty acid composition (Kelsey et al., 2003; Craninx et al., 2008; Samková, 2008; Soyeurt et al., 2008). Kelsey et al. (2003) observed that parity contributes about 10% to the variation in milk FA composition of dairy cows. Studies by Nogalski, et al. 2012 found that Parity had a significant effect on the FA profile of milk fat during the first two months of lactation. Nogalski et al. (2012) also noted that milk fat of primiparous cows had a lower proportion of SCFA and medium-chain fatty acids, and a higher proportion of LCFA, compared with the milk fat of older cows. This was supported by Samková et al. (2012) who also observed that primiparous cows produce milk fat with a higher proportion of USFA and reduced proportions of SFA than multiparous cows. Thomson et al. (2000) also noticed a higher proportion of oleic acid and total USFA in milk fat from primiparous cows compared to multiparous cows. Similarly, Craninx et al. (2008) found lower levels of palmitic acid and higher levels of stearic acid, oleic acid, vaccenic acid and CLA in milk fat of primiparous cows. This difference can be explained paritally by changing milk production and fat content during the individual lactations (Bradford and Allen, 2004). Additionally, the amount of fatty acid synthase participating in fatty acid biosynthesis in the mammary gland is higher in primiparous cows compared to multiparous (Miller et al. (2006). This difference in parity can be explained paritally by changing milk production and fat content during the individual lactations (Bradford and Allen, 2004). Additionally, the amount of fatty acid synthase participating in fatty acid biosynthesis in the mammary gland is higher in primiparous cows compared to multiparous (Miller et al. (2006).

1.6.3 Breed

Milk FA composition is known to vary among individuals of the same breed and between breeds (Schennink et. al 2009). Higher milk CLA and MUFA contents have been reported in Holsteins than in Jerseys when grazed on pasture or fed diets containing conserved forages and grains (Lawless et al., 1999; White et al., 2002). This is probably due in part to a lower *SCD1* or Δ-9

desaturase activity observed in Jersey (Soyeurt et al., 2008). Higher concentrations of SFA, especially of short and medium carbon chains are observed in Jersey milk fat (Arnould and Soyeurt, 2009). The contents of C6:0 to C14:0 are 8 to 42% higher in Jersey as compared to Holstein (Palmquist et al., 1992). In another study, De Peters et al., (1995) observed similar contents of SCFA in the milk of Jersey and Holstein cows (Table 1.2). Beaulieu and Palmquist, (1995) found that Jersey milk fat contained a higher proportion of short and medium chain FA (C4:0, C6:0, C8:0, C10:0, C12:0) and lower proportions of long chain FA (C18:0) than milk fat from Holstein cows. Stull et al., (1964) and Palmquist, (1995) reported that the proportion of medium chain FA (C6:0, C8:0, C10:0, C10:1 and C12:0) was higher while long chain FA (C16:0 and C18:0) was lower in Jersey than in Holstein milk fat. Brown-Swiss milk fat contains a lower amount of C18:3 as compared to Holstein (DePeters et al., 1995). The variation in milk fat composition among dairy breeds could be partially explained by the metabolic process of FA synthesis (Soyeurt et al., 2008). Studies have showed that among all the animal factors affecting milk FA profile, individual variability is the most important one (Khanal and Olson, 2004). The variation among individual cows is related to two factors: (1) ruminal production of transvacenic acid and (2) the activity of lipogenic enzymes (e.g. *SCD1*) (Khanal and Olson, 2004).

Table 1.2 Range of fatty acid composition (g/100 g of total fat) across dairy breeds

(Samková et al., 2012)

Individual Fatty acid	Holstein	Brown swiss	Jersey	Friesian
C4:0	2.7-9.4	3.1-7.7	4.2-5.8	4.2-5.4
C6:0	1.3-2.6	0.7-2.7	2.7-3.4	2.6-3.3
C8:0	0.5-1.3	0.2-1.5	1.5-2.1	1.6-1.9
C10:0	0.9-2.9	0.4-3.5	3.1-4.7	3.0-4.2
C12:0	0.9-3.2	0.4-3.8	3.0-5.2	3.1-4.4
C14:0	4.2-10.9	2.5-10.5	9.6-12.6	8.9-12.6
C14:1 c9	0.1-1.2	0.2-1.1	0.5-1.1	0.5-1.4
C16:0	25.0-33.4	24.4-32.6	23.6-30.3	22.1-32.9
C16:1 c9	0.8-2.4	0.7-1.8	0.7-1.4	0.9-1.5
C18:0	6.6-18.3	8.4-20.7	10.0-14.1	8.7-13.4
C18:1	19.6-30.4	19.4-32.3	17.6-25.0	17.7-28.1
C18:1, t11	0.7-2.4	0.6-1.8	---	---
C18:2, c9,12	2.5-4.4	2.6-4.5	0.9-1.9	1.4-2.3
CLA	0.2-0.7	0.2-0.7	0.6-1.5	1.0-2.4
C18:3, c9,12,15	0.2-0.5	0.3-0.5	0.5-0.9	0.6-1.1

While the rumen output of CLA contributes only marginally to the overall CLA content in milk, it has been observed that individual cows may vary over 3-fold in the activity of *SCD1* (Kelsey et al., 2003; Lock and Garnworthy, 2002; White et al., 2001). Stearoyl-CoA desaturase 1 has been primarily implicated in the variation in milk fat CLA content among individual cows. Animal related factors and their contributions towards the CLA content of milk fat are summarized in table 1.3.

Table 1.3 Animal related factors and their influence on the CLA content of milk fat

Adapted from Khanal and Olsen (2004).

Factor	Effect on Conjugated linoleic acid
Species	Ruminants > Non- ruminants
Breed	Holstein>Brown Swiss> Normandes> Jersey
Stage of lactation	Minimal
Parity	Minimal
Animal to animal	Positive with higher delta-9 desaturase activity

1.6.4 Epigenetic factors

Epigenetic refers to changes in genome function (phenotypes) that occur because of chemical changes in DNA and its associated chromatin. It does not affect DNA sequence (Rakyan et al., 2011; Allis and Jenuwein, 2016). These changes can remain stable through rounds of cell division and even from mother to offspring (i.e. transgenerational; Singh et al., 2012). Whilst factors like breed/genetics, parity, stage of lactation etc. may influence mammary function endogenously, through either up- or down regulation of milk synthesis or mammogenesis, it is now becoming increasingly evident that environmental factors may also result in permanent effects in the offspring. Such environmental factors are regulated through epigenetic mechanisms like DNA methylation (Singh et al., 2010), histone modifications such as acetylation, ubiquitination, methylation, and phosphorylation (Singh et al., 2010). These mechanisms may alter the structures concerned and may either inhibit or increase gene expression (Baird, 2002). Epigenetic mechanisms play a role in the molecular regulation of milk

production in dairy cows and it may be part of the enormously complex regulatory pathways supporting milk production (Singh et al., 2010) and subsequent milk components including milk fat.

Many categories of RNAs have been associated in epigenetic inheritance across generations which includes maternal stores of mRNAs long non-coding RNAs as well as various small RNAs like small interfering RNAs, piwi RNAs, miRNA that interfere with transcription, mRNA stability, or translation (Mercer and Mattick, 2013; Heard and Martienssen, 2014) . Micro RNA base pair with mRNA and by so doing they regulate gene expression in animals through the following ways: translation initiation inhibition, translation elongation inhibition, co-translational protein degradation and premature termination of translation (Huntzinger and Izaurralde, 2011). Additionally, to detecting miRNA expression in the mammary gland, functional studies have directly linked several miRNAs with mammary gland physiology (Ibeagha-Awemu and Zhao, 2015; Ammah, 2018).

Long non-coding RNAs (lncRNAs) non-protein coding transcripts that are longer than 200 nucleotides and regulate gene expression post-transcriptionally by interfering with the microRNA pathways, involving in diverse cellular processes (Cao, 2014). They interact with DNA, RNA, protein molecules and acts as an important regulator in chromatin organisation, post-transcriptional and transcriptional (Cao, 2014).

Alterations in DNA and histone like methylation and modification respectively in animals can also be caused by nutritional changes (Triantaphyllopoulos, 2016). Deoxyribonucleic acid hypermethylation in rat foetus livers have been induced by a protein-deficient diet (Rees et al., 2000). Additionally, fat exposure during development induces persistent changes in hepatic PUFA status in offspring through epigenetic regulation of the FADS2 gene (Mennitti et al., 2015).

1.6.5 Season

Seasonal changes can also affect the composition and content of milk fat. Lock and Garnsworthy (2003) found that the quantity of CLA in milk is higher in the month of May and June when the cows graze on fresh pasture compared to the winter months when they are fed with silage.

Furthermore, they stated that fresh grass increases the synthesis of CLA through the increase in desaturase activities in the mammary gland. Desaturase activities may be dependant on the amount and intake of sugar from the pasture (Rearte, 2005). It has also been suggested that the reduction in CLA in milk during autumn may be as a result of some seasonal variation in C18:3 in the pasture (Zunong et al., 2008). Additionally, seasonal factors like humidity, heat stress and photo-period (light quality and density during sunrise and sunset) influences nutrients and DM intake of pasture (Linnane et al., 2001; West, 2003). This makes it possible that the quality and DM intake of pasture can affect the amount of CLA in milk (Chilliard et al., 2000).

1.6.6 Nutrition

Nutrition is one of the factors that greatly impacts milk fat composition (Brzozowska et al.2018). The largest changes in milk FA composition have been obtained either by changing the amounts and the nature of forages in the diets of cows, particularly pastures, or by adding plant or marine oils to the diet (Dewhurst et al., 2006; Chilliard et al., 2007). Despite their low oil content, forages may contribute 25-35% of the total FA in bovine milk and are the source of acetate and butyrate, which are the precursors for *de novo* synthesis of fatty acids in the mammary gland (Mansbridge and Blake, 1997). It has been shown that by increasing the concentrate ratio of ruminants reduces the C6:0 to C16:0 FA content of milk fat and increases the proportions of C18:1 and C18:2 (Grummer, 1991). This is consistent with a reduction in the supply of acetate and butyrate for *de novo* FA synthesis and a corresponding increase in C18:1 and C18:2 from adipose tissue FA mobilization. Plant products like linseed, soybeans, safflower and sunflower are the most effective sources of unsaturated plant lipids used to enhance CLA and USFA content of milk fat. Marine oil (e.g. fish oil) is more effective than plant oils for enhancing CLA, vaccenic acid (VA) and omega-3 FA in milk fat, especially when fish oil is fed in combination with oil supplements rich in LA (Mele, 2009). Feeding dairy cows with a ration containing 45g/kg (on dry matter basis) of a mixture (1:2 wt/wt) of fish oil and sunflower oil resulted in respectively 810, 1294 and 158% increases in milk fat CLA, VA and long chain omega-3 FA (Jones et al., 2005). Cows fed with sunflower oil showed a larger variation in milk fat CLA content compared to those fed with peanut or LSO (Kelly et al., 1998a). Similarly, there

is a higher variation in milk fat CLA content of individual cows fed diets with a higher concentration of CLA such as all pastures (Kelly et al., 1998b), TMR supplemented with free oil (Kelly et al., 1998a), or a diet with higher forage concentration ratio (Jiang et al., 1996). Milk fat CLA content of cows in confinement and fed total mixed ration has smaller variations compared with milk from cows grazing on pastures (White et al., 2001). Generally, the FA composition of oil seeds varies widely and contains the LCFAs C18:1, C18:2 and C18:3 in varying proportions (Table 1.4). Addition of oils or oilseeds in the diets of dairy cows results in reductions in the levels of *de novo* synthesized FA (C4 to C16) and a concomitant increase in the levels of one or more of the LCFAs C18:0, C18:1, C18:2 or C18:3 (Bauman et al., 2001; Mansbridge and Blake, 1997). A number of authors have observed that fat, oils, and oil seeds that are rich in LA are very effective in increasing milk fat CLA content of cows fed total mixed ration containing 50% forage and 50% concentrate (Whitlock et al., 2003; Ward et al., 2003; Dhiman et al., 2000).

Linseed (also known as flaxseed) oil or cake has been widely used to increase the PUFA content of milk. Linseed generally contains 40% oil, 30% diet fiber, 20% protein, 4% ash and 6% moisture (Wang et al., 2008). Linseed is derived from flax plant (*Linum usitatissimum* L.). When whole linseed or LSO is added to the diet of dairy cows, it increases the ALA content of their milk between 29% and 88% (Chouinard et al., 1998; Loor et al., 2005; Bell et al., 2006; AbuGhazaleh et al., 2003). Increases in the ALA content of milk as high as 2 to 3-fold have been reported when dairy cows were fed raw whole or micronized linseed (Gonthier et al., 2005; Petit, 2002), LSO (Kelly et al., 1998) or milled linseed (Ward et al., 2002).

Safflower oil from the seeds of *Carthamus tinctorius L* is colorless and flavorless dietary oil that is rich in USFA. There are two types of safflower varieties; one type produces oil that is high in oleic acid (about 80 %) (a MUFA) and another type produces oil that is high in LA (about 76 %) (a PUFA) (Table 1.4). The high LA SFO is commonly used as a dietary supplement for animals. Supplementation of sheep diets with 6% SFO (high in LA) resulted in increased levels of USFA and especially CLA in the lean tissue, without adversely affecting growth performance, carcass characteristics, or color stability of lamb (Boles et al., 2005). Similarly,

Kott et al., (2003) reported that feeding safflower seeds to lambs resulted to more than two-fold increases in the concentrations of USFA and *cis*-9, *trans*-11 CLA in muscle tissue as compared to meat from control lambs. Li et al., (2012) suggest that increase in milk CLA by dietary supplementation with SFO might be due to an effect on some mammary lipogenic enzymes like (*SCD, LPL, ACACA, FAS*).

Table 1.4 Fatty acid composition (% of fat) of some dietary ingredients used for enhancing CLA content of milk. Adapted from Khanal and Olsen, (2004)

Dietary ingredient	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
Linseed oil	---	5.6	3.7	17.7	15.4	57.2
Sunflower seed/oil (high oleic acid)	0.1	4.6	2.3	79.3	13.2	0.4
Sunflower oil/seed (high in linoleic acid)	---	5.0	4.0	20.0	69.0	---
Safflower seed/oil (high linolenic acid)	0.1	5.9	2.4	15.2	76.0	0.3
Safflower seed/oil (high oleic acid)	---	8.0	3.0	80.0	---	16
Canola seed/oil	4.1	1.8	---	58.9	22.0	13.2
Palm oil	1.5	43.6	3.2	45.7	2.2	0.2
Corn silage	6.9	16.7	1.9	16.8	54.9	2.7
Pasture grass	0.6	21.0	1.8	2.1	21.1	53.4
Olive oil	---	13.0	2.5	74.0	9.0	---

1.7 Factors that influence the concentration of blood metabolites

Measurement of the metabolic profile of dairy cows is useful because it can be used to assess the nutritional and health states of the dairy herd (Sulieman et al., 2017). However, the levels of these blood metabolites like glucose, NEFA, TAG, and BHBA are influenced by feeding practices, physiological and metabolic states of the cow, breed, and genetics. For example, during transition period (transit from late gestation to early lactation), during which the cow

experiences a negative energy balance, TAG that were stored in the adipose tissue are metabolized as an energy source to compensate for energy deficiency (Gruffat et al.,1996; Herdt, 2000). Triacylglycerides are subsequently hydrolysed into NEFA and glycerol. This leads to a significant release of NEFA into the blood stream and subsequent accumulation of BHBA in the blood (Wankhade et al., 2017) because of intense lipid mobilisation from its tissue stores (Contreras and Sordillo, 2011). This is because of negative energy balance that the animal experienced during the transition period, leading to higher demand for nutrients, energy and reduced feed intake for milk synthesis (Wankhade et al., 2017). Serum NEFA production and circulation during the transition period is mostly influenced by onset of lactogenesis, calving and numerous changes in physiological, metabolic, and endocrine status to support parturition and lactogenesis (Adewuyi et al. 2011). The duration and severity of negative energy balance is noticed by increased circulation of NEFA and BHBA and reduced concentrations of glucose (Drackley, 1999). To alleviate the harmful effects of blood NEFA daily exercise such as walking activity may help to burn excessive NEFA through β-oxidation in the muscles (Adewuyi et al. 2011). Non esterified fatty acids and VLDL values correlate highly in dairy cattle with lower parity because their livers can easily process in coming NEFA to VLDL and eventually secrete it into the blood stream (Oikawa et al., 2017). Excessive accumulation of fat in the liver impairs its normal function (Murondoti et al. 2004) and this may lead to hyperketonemia (Herdt, 2000). Blood glucose levels of lactating dairy cows undergo increased demand during the early postpartum period to meet the requirements for milk production since glucose is a key component of cow's milk and regulates the concentrations of other blood metabolites (Lucy, 2001). Blood glucose levels of dairy cows are influenced by breed, country and, geographical location (Kappel et al., 1984; Mapfumo and Muchenje, 2015).

1.8 Dietary regulation of fatty acid metabolism

Dietary lipids are extensively altered by bacterial metabolism in the rumen with marked effects on bacterial populations (e.g. *Butyrivibrio fibrisolvens*) and rumen microbial processes. This has a major effect on the fat content and fatty acid composition of milk (Bauman and Griinari, 2003). Certain diets, especially those rich in PUFAs cause a marked reduction in the milk fat yield of

dairy cows (Bauman and Griinari, 2003). This is commonly referred to as MFD. Milk fat depressioon was first described by a French scientist, Boussingault in 1845 (Bauman et al., 2011) and it is characterised by a reduction of up to 50% in milk fat yield with no change in the yield of milk and other milk components (Bauman et al., 2011). There are several theories that have been proposed to explain diet induced MFD (Shingfield et al., 2010; Bauman et al., 2011). One of these theories of MFD attributed the decreases in milk fat synthesis to reductions in the supply of acetate and 3-hydroxy-butyrate for *de novo* fatty acid synthesis in the mammary gland. The second theory speculates that there is elevated insulin secretion, stimulating the preferential partitioning of fatty acid towards adipose tissue at the expense of the mammary gland hence reduced fat secretion in milk. The third theory points to direct inhibition of mammary lipogenesis by *trans* fatty acids formed during the biohydrogenation of dietary USFA in the rumen (Figure 1.5). Of all these hypotheses, the biohydrogenation theory appears to offer a more convincing explanation for MFD. This is supported by the fact that associated increase in milk *trans*-10, C18:1 and *trans*-10, *cis*-12 CLA (Figure 1.5) isomers during diet-induced MFD inhibits milk fat synthesis (Griinari et al., 1998; Piperova et al., 2000; Baumgard et al., 2000) (Figure 1.5). The biohydrogenation theory states that 'under certain dietary conditions, the pathways of biohydrogenation are altered to produce unique fatty acid intermediates which are potent inhibitors of milk fat synthesis (Shingfield et al., 2010; Bauman et al., 2011). Figure 1.5 shows a generalized scheme of rumen biohydrogenation of LA under normal conditions and during diet-induced MFD. Changes in rumen biohydrogenation of USFA and the passage of specific intermediates of biohydrogenation out of the rumen (e.g. *trans*-10, *cis*-12 CLA) subsequently reduces milk fat synthesis in the mammary gland by altering expression of genes involved in fat synthesis (Bauman et al., 2011). Diets causing MFD can be divided into two groups (Davis and Brown, 1970). The first group are diets which provide large amounts of readily digestible carbohydrates and reduced amount of fibrous components. The most common being a high grain/low-roughage diet. Diets with an adequate source of grounded or pelleted fiber also fall into this group because these processes reduce the ability of fiber to maintain normal rumen function (Bauman and Griinari, 2003). The second group of diets that can induce MFD are dietary supplements containing USFA like plant and marine oils (e.g. fish oil, LSO, SFO, soybean oil, marine algal lipids, etc.) (Bauman and Griinari, 2001, 2003; Bauman et al.,

2011). The prerequisite for MFD is the presence of USFA and also low fiber diets (Griinari et al., 1998). Plant oil supplements will not lead to MFD if roughage intake is high or the effectiveness of roughage fiber is sufficient to maintain normal rumen function (Brown et al., 1962; Kalscheur et al., 1997).

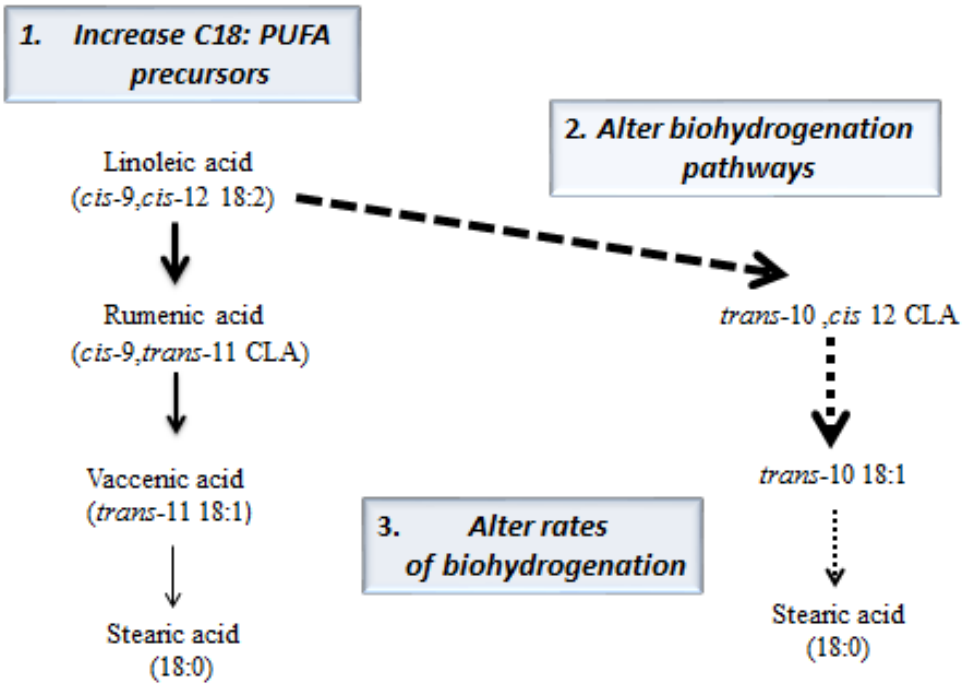

Figure 1.5 Generalized scheme of ruminal biohydrogenation of linoleic acid under normal conditions (left side) and during diet-induced milk fat depression (dotted lines, right side). The grey boxes highlight three potential means by which dietary components can increase the risk of MFD. A shift in the biohydrogenation pathway as a result of nutrients rich in USFA lead to the production of *trans* 10, *cis* 12 CLA which is responsible for MFD. Adapted from Lock, (2010).

Fish oil and oils from marine mammals and marine algae are characterized by the presence of two PUFA: eicosapentaenoic acid (C20:5) and docosahexanoic acid (C22:6). In contrast to plant oils, fish oils and oils from marine mammals will induce MFD even when diets contain an adequate level of effective fiber (Bauman and Griinari, 2003). Most studies that investigated the effect of CLA during lactation in dairy cows consistently demonstrated inhibitory effects on milk fat while milk yield and other milk components are generally unaffected (Bauman and Grinarii, 2003). Baumgard et al., (2000) showed that reduction in milk fat secretion reaches a lowest point by 4 to 5 days of supplementation and returns to previous levels when CLA treatment is terminated.

Most studies have lasted a few days, but long-term studies for about 20 weeks indicated that the reduction in milk fat persists throughout the TP (Perfield et al. 2002; Bernal-Santos et al., 2003). Treatment covering all phases of the lactation cycle showed no adverse effects on animal health and well-being (Perfield et al., 2002; Castaneda-Gutierrez et al., 2005).

1.9 Molecular mechanisms of milk fat depression

During MFD, lipogenic capacity and transcription of key mammary lipogenic genes are coordinately down-regulated (Bauman et al., 2011). Synthesis of milk fat requires coordination of enzymes involved in metabolite transport, *de novo* lipogenesis, fatty acid transport, fatty acid desaturation, esterification, and the formation, transport, and excretion of the milk lipid droplet. The fact that *trans*-10, *cis*-12 CLA-induced and diet-induced MFD involve a decrease in milk fat output of both *de novo* and preformed fatty acid suggests a coordinated regulation of enzymes of lipid synthesis (Bauman et al., 2011). Coordinated suppression of mammary lipogenic genes suggests involvement of a central regulator of lipid synthesis. Messenger ribonucleic acid (mRNA) abundance of fatty acid synthase (*FASN*), acetyl-CoA carboxylase alpha (*ACACA*), lipoprotein lipase (*LPL*), stearoyl CoA desaturase 1 (*SCD1*), fatty acyl-CoA ligase (*FACL*), glycerol-phosphate-acyl transferase mitochondria (*GPAM*), and 1-acyl-glycerol-3-phosphate-acyltransferase (*AGPAT*) have been shown to decrease during MFD (Bauman et al., 2011).

Peterson et al. (2004) reported a decrease in the activities of stearoyl response element binding protein 1 (*SREBP1*) during *trans*-10, *cis*-12 CLA treatment of bovine mammary epithelial cells. In support of this, Mach et al., (2011) reported that the most prominent functional characteristic of lipid metabolism category was the down regulation of the TF *SREBP1*, when cows were supplemented with dietary unprotected USFA. This suggests that an increase in LCFA and *trans*-fatty acids reaching mammary gland from blood may affect expression of key transcription regulator genes and their response genes. Acetyl carboxylase A was also shown to be down regulated with *SREBP1*, which catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA (Bernard et al., 2008), during supplementation with USFA. Mammary expression of *SREBP1*, a member of a family of TF that play critical roles in the regulation of lipogenesis, has been reported to decrease in similar magnitude as milk fat yield during both diet and *trans*-10, *cis*-12 CLA-induced MFD (Gervais et al., 2009; Harvatine and Bauman, 2006). Consequently, the key lipid synthesis enzymes that are down-regulated during *trans*-10, *cis*-12 CLA-induced and diet-induced MFD contain a stearoyl response element in their promoter and are known to be regulated by *SREBP1c* (Harvatine and Bauman, 2006). Spot 14 (S14) is a *SREBP1*-responsive gene that encodes a nuclear protein that is closely associated with the regulation of fatty acid synthesis in lipogenic tissues including lactating mammary tissue (Cunningham et al., 1998). Mammary expression of S14 in lactating cows is down-regulated during diet-induced MFD and *trans*-10, *cis*-12 CLA treatment (Harvatine and Bauman, 2006).

1.10 Mammary gland transcript profiling

Several methods have been used to profile mammary gland transcriptome after dietary supplementation with USFA. These methods include quantitative real-time polymerase chain reaction (qPCR), microarray and RNA-sequencing (RNA-Seq) (the most robust transcript profiling method). Transcriptome signifies the complete set of transcripts in a cell and encompasses a multitude of coding and non-coding RNA species such as mRNA, rRNA, tRNA and small RNAs (e.g. microRNA, piwi RNA, small nucleolar RNA, circular RNA, promoter-associated RNAs, antisense 3′termini-associated RNA, etc). Their understanding is essential for interpreting the functional elements of the genome and revealing the molecular constituents of

cells and tissues, and also for understanding development of diseases (Wang et al., 2009). The main objective of transcriptomics therefore is to register all expressed transcripts of a cell or an organ under different conditions. Transcriptomics also determines the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing patterns and other post-transcriptional modifications. It is also used to quantify the changing expression levels of each transcript during its development and under different conditions (Wang et al., 2009).

1.10.1 Real time quantitative PCR (qPCR)

This technique of profiling gene expression is based on the revolutionary method of PCR, developed by Kary Mullis in the 1980s. The advantages of qPCR are that they have enormous sensitivity, are very precise, and provides "real-time" monitoring of PCR products as they are generated. This technique has been used to describe the effects of dietary USFA on the expression of a number of candidate genes in mammary gland involved in lipid metabolism (Harvatine and Bauman, 2006; Bauman et al., 2008; Bionaz and Loor, 2008a and 2008b; Kadegowda et al., 2009; Jacobs et al., 2011). Using qPCR, Jacobs et al., (2011) showed that mammary *SCD1* expression was reduced by supplementation of soybean oil (2.7%) compared with rapeseed oil (2.7%) and LSO (2.7%). Bionaz and Loor, (2008) used qPCR to study mammary tissue mRNA expression of 45 genes associated with lipid synthesis and secretion from the late pre-partum through the end of subsequent lactation. They demonstrated the involvement of several lipogenic genes and their levels of transcript abundance and also their interactions (pathways) throughout the lactation cycle. However, this method is limited as compared to next generation sequencing of the transcriptome because it relies on prior knowledge of genes and moreover, only a few genes can be measured at a time. It may be difficult therefore to profile the complete transcriptome with this technique.

1.10.2 Microarray

This is a hybridization-based method to profile gene expression. It typically involves incubating fluorescently labelled complementary DNA (*cDNA*) with custom-made microarrays or commercial high-density oligo microarrays. Microarray technique has the advantage over qPCR in that it provides the ability to monitor simultaneously expression of multitude of genes and to discover target genes that would not have been detected by a more focussed technique like qPCR (Sinicropi et al., 2007). Only one study (Mach et al., 2011) has used this technique to determine the effects of supplementing unprotected dietary USFA from different plant oils (LSO, soybean oil and rapeseed) on gene expression in the mammary glands of dairy cows. They reported a robust transcriptional adaptation to diet with 972 genes affected, suggesting a strong impact on metabolism and other cellular functions in the mammary gland. This technique, as compared to previous studies employing qPCR, provided a deeper holistic view of the genes that are expressed and the molecular events that occur when the mammary gland adapts to changes in the supply of dietary lipids, and consequent changes in milk yield and composition. However, this technique offered a limited ability to fully catalogue and quantifies the diverse RNA molecules that responded to the effect of diet because it is restricted to the targets on the array.

1.10.3 RNA-Seq

Since its initial application, RNA-Seq or massively parallel *cDNA* sequencing has permitted many advances in the characterization and quantification of transcriptomes (Ozsolak and Milos, 2011). RNA-Seq uses recently developed deep sequencing technologies and it is the first sequencing-based method that allows the entire transcriptome to be surveyed in a very high-throughput and quantitative manner (Wang et al., 2009). Through this method, we now have a new appreciation of the complexity of the transcriptome, including a multitude of previously unknown coding and non-coding RNA species (e.g. microRNAs, promoter-associated RNAs and antisense 3′ termini-associated RNA, etc.) (Berretta and Morillon, 2009; Kapranov et al., 2007). RNA-Seq technology offers several advantages over existing technologies. Firstly, unlike hybridisation-based techniques, RNA-Seq is not limited to detecting transcripts that

correspond to existing genomic sequences or to the number of targets on the array. This property makes RNA-Seq particularly attractive for non-model organisms with genomic sequences that are yet to be determined. RNA-Seq can disclose the precise location of transcription boundaries right to a single base resolution, allow the identification of transcription start sites, the identification of new splicing variants, the monitoring of allele expression, allows for the precise quantification of exon expression and splicing variants (Cloonan et al., 2008; Morin et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2008; Shendure, 2008; Wilhelm et al., 2008; Wang et al., 2009; Tsuchihara et al., 2009). In addition, RNA-Seq can also reveal sequence variations (for example, SNPs) in the transcribed regions (Cloonan et al., 2008; Morin et al., 2008). The results of RNA-Seq also show high levels of reproducibility, for both technical and biological replicates (Nagalakshmi et al., 2008; Cloonan et al., 2008; Morin et al., 2008). These characteristics make RNA-Seq useful for studying complex transcriptomes (Wang et al., 2009).

1.11 miRNA and miRNA-mRNA co-expression analysis

Target genes that are involved in many biological processes, and that may play roles in the regulation of milk and blood components are regulated by many factors including microRNAs (miRNAs) and small interfering ribonucleic acid (siRNA), RNA transcriptional control, RNA processing control, protein activities control, RNA transport control. Assessments of individual gene expression cannot alone explain the complex etiology of milk fat traits; hence an integrative approach in assessing gene expression in a network basis is necessary to unravel the molecular mechanism underlying milk fat traits. Messenger ribonucleic acid and miRNA expression data (microarray or RNA-Sequencing) have been used widely to investigate the relationship between miRNAs and their target genes (Freiesleben et al., 2016). Microribonucleic acid, which are non-coding RNAs are about 20 to 24 nucleotides long and can post-transcriptionally suppress target gene expression by recognizing the complementary target sites in the 3' untranslated region of mRNAs (Bartel et al., 2004). Target mRNA sequences are perfectly or partially complemented by miRNAs, leading to degradation or suppression of transcribed mRNAs (Lewis et al., 2005). Additionally, miRNAs and their target genes have a complex relationship as multiple miRNAs can target multiple mRNAs (Lim et al., 2005; Krek

et al., 2005). Network analysis has been used to dissect many complex traits (Zhang et al.,2018; Duy et al., 2017; Chen et al., 2008; Gargalovic et al., 2006). The weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) groups genes into modules based on their co-expression pattern across a set of samples and then relates these modules to the traits of interests in order to reveal biological relevant modules or clusters (Seo et al., 2017).

The weighted gene co-expression network analysis has been used to detect modules (expression patterns of miRNA during lactation (Do et al., 2017) and to identify miRNA regulatory mechanisms involved in milk yield and components traits fat, protein, somatic cell count (SCC), lactose, and MUN through miRNA target gene enrichment analysis (Do et al., 2017).

There are three main steps that are involved in WGCNA. The first step involves the identification of individual relationships between genes based on correlation measures (Steuer et al., 2002; Margolin et al., 2006) between each pairs of genes. The Pearson's or Spearman's correlation measures have been used to construct networks (Figure 1.6).

Secondly, a network is constructed from the co-expression associations where each node represents a gene and each edge represents the strength of the co-expression relationship (Albert et al. 2002).

In the third step, modules (groups of co-expressed genes) are identified using one of the available clustering techniques (including k-means clustering or hierarchal clustering). In clustering, genes with similar expression pattern across different samples are grouped rather than only pairs (Seo et al., 2017). Functional enrichment analysis is subsequently carried out on modules to identify and rank over represented functional categories in a list of genes (Gupta et al., 2014; de Magalhaes et al., 2010).

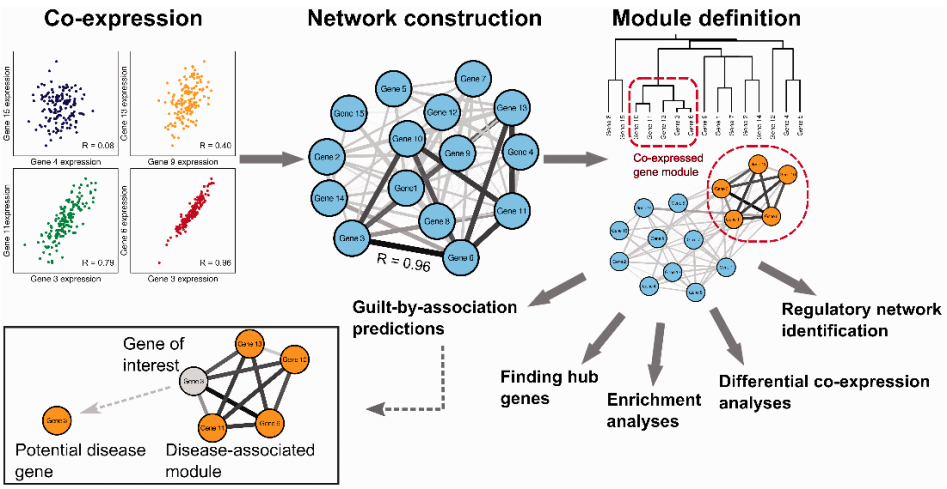

Figure 1.6. An example of a co-expression network analysis. First, pairwise correlation is determined for each possible gene pair in the expression data. These pairwise correlations can then be represented as a network. Modules or clusters within these networks are defined using clustering analysis. The network and modules can be interrogated to identify regulators, functional enrichment and hub genes. Differential co-expression analysis can be used to identify modules that behave differently under different conditions. Adapted from Seo et al., (2017).

1.12 Strategies to alter milk fatty acid profiles

1.12.1 Nutritional strategies

The application of feeding strategies is undoubtedly the most efficient way to modify milk fatty acid composition in order to enhance the healthfulness of dairy products. Consequently, several studies in the last three decades have employed different nutritional strategies to alter the composition of milk fatty acids in favor of health promoting isomers (Bauman and Griinari, 2003; Chilliard et al., 2007; Shingfield et al., 2010). Mach et al., (2011) supplemented the diets of 28 mid-lactation Holstein-Friesian cows with 2.7% rapeseed oil, 2.7% soybean oil, 2.7% LSO or a mixture of the three oils and observed a decrease in the proportion of *de novo* fatty acids whereas that of LCFA increased. Their study also recorded an increase in milk yield but a

decrease in milk fat and protein percentages. Similarly, Bu et al., (2007) examined the effectiveness of linoleic or linolenic rich oils to enhance C18:2, *cis*-9, *trans*-11 CLA, and C18:1, *trans*-11vaccenic acid in milk. They concluded that feeding cows with soybean or flaxseed oil resulted in increased proportions of milk vaccenic acid and CLA and consequently milk with a higher nutritive and therapeutic value. In another study, Yarahmadi et al. (2012) noted significant enrichment in milk USFA content when the diets of high yielding lactating dairy cows were supplemented with 2% soybean oil, 2% sunflower oil or 2% canola oil. Furthermore, Hurtaud and Peyraud (2007) examined the effects of feeding cows with linolenic acid rich cruciferous plant camelina on the fatty acid composition of dairy products and the properties of butter. They noted that feeding the same amount of PUFA to cows either through camelina seed (630g/d, CS) or camelina meal (2kg/d) generated a greater proportion of MUFA, notably C18:1 *trans* isomers (*trans*-10 and *trans*-11 C18:1), which increased by 11.0 and 2.6-fold, respectively, with the camelina meal diet. Camelina also led to an increase in CLA, particularly rumenic acid, *cis*-9, *trans*-11 C18:2 which lead Hurtaud and Peyraud (2007) to conclude that feeding camelina can modify milk fatty acid profile and butter spreadability. Kliem et al., (2011) investigated the effect of replacing calcium salts of palm oil distillate with incremental amounts of conventional or high oleic acid milled rapeseed on milk fatty acid composition in cows fed maize silage-based diets and found that this nutritional strategy lowered milk SFA content without inducing adverse effects on DM intake and milk production. Using fish oil supplementation, Abu-Ghazaleh et al., (2003) reported increased concentrations of *cis*-9, *trans*-11 CLA and TVA in milk fat as compared to control diet (without fish oil). In a recent study, Benchaar et al. (2012) concluded that LSO can be safely supplemented up to 4% in forage-based diets of dairy cows to enrich milk with potential health beneficial fatty acids without causing any detrimental effects on rumen function, digestion, and milk production. Furthermore, Boles et al. (2005) reported that supplementation of sheep diets with SFO of up to 6% of the diet, resulted in increased concentrations of USFA and CLA in the lean tissue, without adversely affecting growth performance, carcass characteristics, or color stability of lamb. In another study, Bell et al. (2006) demonstrated that a combination of SFO with monensin was effective at increasing milk fat CLA. Safflower oil was successfully used to increase the CLA content in tissues of lamb when supplemented with lamb feedlot diets (Mir et al., 2000). The nutritional strategy is

effective but also comes with significant additional production cost. Genetic strategy is a complementatry approach which consist in chosing gene variants with increased desaturation activities in breeding strategy.

1.12.2 Genetic strategies

The influence of individual as well as breed variations on milk fatty acid profiles indicate that these traits can be improved through genetics. Furthermore, reported moderate heritability estimates for individual milk fatty acids (Stoop et al., 2008; Bobe et al., 2008; Garnsworthy et al., 2010) suggest that genetic variability of fatty acids can enable improvement of milk fatty acid composition through breeding. Consequently, numerous investigations including candidate gene as well as genome wide studies around the world have associated SNPs and genomic regions or quantitative trait loci (QTL) with different milk fatty acids (Grisart et al., 2002; Mele et al., 2007; Morris et al., 2007; Schennink et al., 2008; Kgwatalala et al., 2009a, b, c; Conte et al., 2010; Bouman et al., 2011; Ibeagha-Awemu et al., 2012, Ibeagha-Awemu et al., 2016; Duchemin et al., 2017; Knutsen et al., 2018). Candidate gene studies have shown that polymorphisms (SNPs) within the key lipogenic genes *SCD1, FASN, DGAT1, SREBP1, ACACA, GPAT, AGPAT* are associated to variations in milk fatty acid content (Medrano et al., 1999; Taniguchi et al., 2004; Roy et al., 2006, Mele et al., 2007; Moioli et al., 2007; Schennink et al., 2007, 2008; Kgwatalala et al., 2009; Conte et al., 2010). Similarly, genome wide studies based on thousands of SNPs spread over the genome have reported significant as well as suggestive QTLs for milk fatty acids on all bovine chromosomes (Schennink et al., 2009; Stoop et al., 2009b; Morris et al., 2007, 2010; Bouwman et al., 2011, 2012; Wang et al., 2012). However, only a small proportion of the SNPs tested in Genome wide association studies as well as candidate gene studies have significant associations with trait, each explaining only a small part of the variance while the remainder of the genetic variation remains elusive. It is expected that one to a few SNPs contributing the most variance in trait are actually responsible for observed effects. For example, a QTL for milk yield and composition on BTA 14 has been reported consistently in several dairy breeds (Coppieters et al., 1998; Heyden et al., 1999). Using positional candidate cloning technique, Grisart et al., (2002) identified a non-conservative

K232A substitution in the *DGATI* protein which accounted for 10 and 60% of the genetic variance in protein yield and fat percentage, respectively and proposed this gene as the likely candidate for this QTL. This proposition was further supported by the activity of the gene (catalyzes the last step in TAG synthesis) and the fact that mice with a knock-out mutation for this gene do not lactate (Cases et al., 1998; Smith et al., 2000). Using genetic and functional studies, Grisart et al., (2004) provided confirmation of the causality of the *DGAT1* K232A mutation as a quantitative trait nucleotide (QTN) affecting milk yield and composition in dairy cows. Another validated QTN with major effects on milk fat traits (milk yield and composition) is the Y581S mutation in the *ABCG2* (ATP-binding cassette, sub-family G [WHITE], member 2) protein (Cohen-Zinder *et al.* 2005; Olsen *et al.* 2007). The effect of this SNP has been validated in different breeds (Braunschweig, 2010). To successfully implement QTL information in selection programs, it is imperative that the associated SNPs with large effects on traits be validated as QTN(s).

1.13 GENERAL HYPOTHESIS AND OBJECTIVES

My Ph.D. research aimed to understand the effects of diets rich in USFA on blood and milk metabolites of dairy cows (manuscript 1) and to examine the major regulatory mechanisms (highly connected miRNAs and miRNA-mRNA co-expressed pairs) involved in the regulation of the expression of blood metabolites and milk components following dietary supplementation with USFA (manuscript 2). In the last section of my Ph.D. studies, I studied the association of SNP in lipogenic genes with milk *de novo* synthesized fatty acid profiles of Canadian Holstein cows (manuscript 3).

Although numerous investigations have considered the effects of dietary supplementation with USFA including LSO and SFO on milk and blood components, it is not clear what residual effects these nutrients have on the physiology of the animal including blood metabolites and milk components or for how long effects are active after withdrawal of treatment. Furthermore, the mechanisms regulating mammary gland's response to dietary USA are poorly understood.

Therefore, I hypothesized that dietary supplementation with USFA impacts ruminal activities which influences blood metabolites and milk components, and that the residual effects on the physiology of the animal and on blood and milk parameters are longer than current knowledge predicts. In the first part of my PhD study, I examined the treatment and post treatment effects of dietary supplementation of cows' diets with 5% LSO or 5% SFO on milk composition and blood metabolites of lactating Holstein cows. The results from this study are presented in chapter 2 (manuscript 1).

Transcriptomic analysis through next generation sequencing of mammary biopsies from the cows (diets supplemented with 5% [SFO] or 5% [LSO]) revealed mRNA (Ibeagha-Awemu et al., 2016) and miRNA (Li et al., 2015) that were differentially expressed. Since a network of genes and regulatory factors work in concert to influence the phenotypic expression of traits, assessment of gene expression without taking into account the factors that regulate their activities may not adequately explain the complex biological mechanisms underlying the expression of traits. Micro ribonucleic acids interact with mRNA to regulate their expression and consequently biological processes, so it is important to study their synergistic effects on the phenotypic expression of traits. I therefore hypothesized that, factors that regulate gene expression, like miRNA, are involved in cow's response to dietary USFA, which subsequently influenced phenotypic expression of traits (blood metabolites and milk components). Therefore, understanding the functions of highly interconnected miRNA (miRNA modules) and miRNA-mRNA co-expressed pairs in the mammary gland and how they regulate blood and milk phenotypes following dietary supplementation with 5% LSO or 5% SFO was the objective of the second part of my PhD studies and the results are presented in chapter 3 (manuscript 2) of this thesis.

Dietary supplementation with 5% LSO or 5% SFO decreased milk fat percentage by 30.38 % and 32.42 % respectively with a greater impact (reduction) on individual milk fatty acids synthesized *de novo* in the mammary gland (Ibeagha-Awemu et al. 2016). Furthermore, pathways analysis of gene expression data revealed eighteen differentially expressed genes of the fatty acid and lipid biosynthesis pathways (Ibeagha-Awemu et al., 2016). I hypothesized that

polymorphism in the affected genes may influence *de novo* synthesized fatty acid profiles and could serve as biomarkers for breeding for a healthier milk profile for human benefit. Hence, the last objective of my PhD study was to investigate the relationship between SNPs in these lipogenic genes and *de novo* synthesized milk fatty acid composition in Canadian Holstein population. The results of this study have been presented in chapter 4 (manuscript 3) of this thesis.

The results of the research described in this thesis have provided answers to some of the questions raised above as well as provided a better understanding of the effects of diets rich in USFA on milk and blood metabolites; the relationship between SNPs in some genes in the fatty acid and lipid biosynthesis pathways and milk *de novo* synthesized fatty acids and finally, the regulatory (molecular) mechanisms (miRNA regulation) involved in the bovine mammary gland response to dietary supplementation with USFA.

CHAPTER 2

Effects of diets rich in unsaturated fatty acids on blood and milk metabolites in Canadian Holstein cows

Article Description

In this manuscript, I examined the treatment and post-treatment effects of dietary supplementation with 5% SFO or 5% LSO on milk components and blood metabolites of Canadian Holstein cows. Numerous investigations have considered the effects of dietary supplementation with USFA including LSO and SFO on milk and blood components. However, the residual effects of these nutrients on the physiology of the animal including blood metabolites (TAG, NEFA, glucose, BHBA) and milk components (SCC, fat, lactose, protein, MUN, milk yield) or for how long effects are active after withdrawal of treatment is not very clear. We supplemented the diets of dairy cows with 5% LSO or 5% SFO for 28 days. Before this supplemental period, all the animals were under a control diets of total mixed ration of corn and grass silage (50:50) for 28 days. The animals were returned to the control diets for another 28 days after the supplemental period. Blood and milk samples were collected weekly. Daily feed intake was monitored, and body weight was measured at the end of each period. We confirmed that USFA (5% SFO or 5% LSO) influenced blood and milk components during the TP. We further demonstrated that the residual effects of feeding LSO or SFO on the physiology of cows were still active up to three weeks after cessation of treatments.

The results of this part of my project have been published in the Journal of Applied Animal Research with the following citation:

Ammah, A. A., C. Benchaar, N. Bissonnette, N. Gévry, and E. M. Ibeagha-Awemu. 2018. Treatment and post-treatment effects of dietary supplementation with safflower oil and linseed oil on milk components and blood metabolites of Canadian Holstein cows. Journal of Applied Animal Research 46(1):898-906. doi: 10.1080/09712119.2017.1422256**)**

Author Contributions

Conception and design of the study: Eveline Ibeagha-Awemu; Provided inputs on study design: Adolf Ammah, Nathalie Bissonnette and Eveline Ibeagha-Awemu; Data collection: Adolf Ammah.; Data analysis: Adolf Ammah and Eveline Ibeagha-Awemu**;** Interpretation of data: Adolf Ammah, Eveline Ibeagha-Awemu; Drafting of manuscript: Adolf Ammah ; Critical revision of the manuscript: E.M.I.-A and Chaouki Benchaar; Revised and approved the final manuscript: All authors.

Treatment and post-treatment effects of dietary supplementation with safflower oil and linseed oil on milk components and blood metabolites of Canadian Holstein cows

Adolf A. Ammah[a,b], Chaouki Benchaar[a], Nathalie Bissonnette [a], Nicolas Gévry[b] and Eveline M. Ibeagha-Awemu [a]*

[a]Agriculture and Agri-Food Canada, Sherbrooke Research and Development Centre, Sherbrooke, QC, J1M 0C8 Canada.

[b] Département de biologie, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, QC, J1K 2R1, Canada.

***Corresponding author: Eveline.Ibeagha-Awemu@AGR.GC.CA**

Key words: Blood metabolites; milk components; linseed oil; safflower oil; milk fat; Canadian Holstein cow, treatment period, control period, post treatment period, body weight, non-esterified fatty acids, triacylcglycrides, betahydroxybutric acid, mulk urea nitrogen, conjugated linoleic acid, unsaturated fatty acid, alpha linolenic acid, total mixed ration, days in milk, dry matter.

2.1 Abstract

This study investigated the treatment and residual effects of LSO or SFO on milk and blood metabolites of cows. Twenty six Canadian Holstein cows were assigned equally to one of two treatments: a control diet + 5% SFO or 5% LSO for 28 days (TP). The TP was preceded by a control period (CP) of 28 days (all animals on control diet). After treatment, animals were returned to control diet for 28 days (PTP). Blood and milk samples were collected weekly. Daily feed intake was monitored. Body weight (BW) was measured at the end of each period. Feed intake decreased with LSO and SFO ($P<0.05$) while BW increased steadily ($P<0.0001$) throughout. Non-esterified fatty acid and TAG increased ($P<0.0001$) during treatments as compared to control peroid. Beta hydroxybutyric acid increased ($P<0.0001$) while lactose decreased ($P<0.0001$) with SFO only as compard to control peroid. Milk urea nitrogen decreased ($P<0.0001$) with both supplements. Milk fat content reduced ($P<0.0001$) by 34.2% (LSO) and 29.9% (SFO) during TP. Non-esterified fatty acids / MUN and milk fat content returned to control levels one week or three weeks after treatment, respectively, while TAG did not return to initial concentrations by the end of PTP. Our data shows that the residual effects of feeding LSO or SFO on the physiology of cows were still active up to three weeks after cessation of treatments.

Key words: Blood metabolites; milk components; linseed oil; safflower oil; milk fat; Canadian Holstein cow, treatment period, control period, post treatment period, body weight, non-esterified fatty acids, triacylcglycrides, betahydroxybutric acid, mulk urea nitrogen, conjugated linoleic acid, unsaturated fatty acid, alpha linolenic acid, total mixed ration, days in milk, dry matter.

2.1 Résumé

Cette étude a porté sur le traitement et les effets résiduels du LSO ou de l'OFS sur le lait et les métabolites sanguins des vaches. 26 vaches Holstein canadiennes ont été affectées uniformément à l'un des deux traitements: un régime de contrôle + 5% SFO ou 5%LSO pendant 28 jours (période de traitement, TP). Le TP a été précédé d'une période de contrôle (CP) de 28 jours (tous les animaux sous régime de contrôle). Après le traitement, les animaux sont retournés au régime de contrôle pendant 28 jours (période de post-traitement, PTP). Des prélèvements de sang et de lait ont été effectués chaque semaine. L'ingestion quotidienne d'aliments a été surveillée. Le poids corporel (BW) a été pris à la fin de chaque période. L'ingestion d'aliments a diminué avec LSO et SFO ($P<0,05$) alors que BW a augmenté régulièrement ($P<0,0001$). Les acides gras non estérifiés et le TAG ont augmenté ($P<0,0001$) pendant les traitements. L'acide bêta-hydroxybutyrique a augmenté ($P<0,0001$) tandis que le lactose a diminué ($P<0,0001$) avec SFO seulement. L'azote uréique du lait a diminué ($P<0,0001$) avec les deux suppléments. Le taux de matière grasse du lait a diminué ($P<0,0001$) de 34,2 % (LSO) et de 29,9 % (SFO) pendant la TP. Les acides gras non estérifiés / MUN et le taux de matières grasses du lait sont revenus aux niveaux de contrôle une semaine ou trois semaines après le traitement, respectivement, tandis que le TAG n'est pas revenu aux concentrations initiales à la fin de la PTP. Nos données montrent que les effets résiduels de l'alimentation LSO ou SFO sur la physiologie des vaches étaient encore actifs jusqu'à trois semaines après l'arrêt des traitements.

Mots clés: métabolites sanguins, composants du lait, l'huile de lin, l'huile de carthame, la matière grasse du lait, Vache Holstein canadienne, période de traitement, période de contrôle, période post-traitement, poids corporel, acides gras non estérifiés, triacyliques, acide bétahydroxybutrique, azote d'urée de mulk, acide linoléique conjugué, acide gras insaturé, acide alpha-linolénique, ration totale mélangée, jours lait, matière sèche.

2.2 Introduction

Dietary unsaturated fatty acids (USFA) may modulate the metabolism of dairy cows and hence influence the levels of some blood metabolites like glucose, NEFA, TAG, BHBA and milk components like fat, protein, urea nitrogen and lactose. Non-esterified fatty acids are one of the most important metabolites in the blood of dairy cows, especially in the transition period (3 weeks before and 3 weeks after parturition) (Li et al., 2013). Elevated concentrations of NEFA and BHBA have been used as indicators of excessive negative energy balance associated with increasing risk of diseases during the transition period in dairy cows (LeBlanc et al., 2005; Chapinal et al., 2011).

The origin of MUN is mainly from excess ammonia that is released from dietary protein degradation in the rumen or from deamination of excess dietary amino acids. Milk urea nitrogen is routinely used in dairy herds as a non-invasive measurement to monitor the protein status of animals and the efficiency of nitrogen utilization (Broderick and Clayton, 1997; Jonker et al., 1998). Milk lactose determines milk osmolality and its synthesis determines the rate of water secretion in milk (Miglior et al., 2006). It should be noted that the water content of milk largely determines milk fat and protein concentrations (Henao-Velásquez et al., 2014).

Materials rich in USFA have been shown to influence blood glucose and NEFA levels in dairy cows (Salehi et al., 2015). Supplementing the diets of periparturient cows with CLA (600 g/d of rumen inserted-CLA) decreased blood NEFA concentration while glucose was increased during the first week of lactation (Odens et al., 2007). Increased NEFA levels in blood plasma of cows fed diets supplemented with flaxseed (linseed) and fish oil have been reported (Drackley, 1999; Gonthier et al., 2005; Petit et al., 2002).

Oil and seeds from plant products like linseed, soybean, safflower, sunflower and fish oil with high concentrations of USFA have been used to enhance the milk content of beneficial fatty acids such as CLA, n-3 and n-6 fatty acid (Shingfield and Griinari 2007; Shingfield et al., 2010). Linseed oil is rich in alpha-linolenic acid (ALA, an n-3 fatty acid), about 52–63% of total fat (Suksombat et al., 2013) thus making it an attractive fat supplement in ruminant diets. Safflower

oil from the seeds of Carthamus tinctorius L (Yenice variety) is colourless and flavourless and rich in linoleic acid (about 76% of total fat, a PUFA (He and Armentano 2011).

Although numerous investigations have considered the effects of dietary supplementation with USFA including LSO and SFO on milk and blood components, it is not clear what residual effects these nutrients have on the physiology of the animal or for how long effects are active after withdrawal of treatment. In this study, we examined the treatment and post-treatment effects of dietary supplementation of cows' diets with 5% LSO or 5% SFO on milk composition and blood metabolites of lactating Holstein cows.

2.3 Materials and Methods

2.3.1 Experimental animals and diets

Twenty-Six Canadian Holstein cows (BW = 676.6 ± 25.9 kg; milk yield = 35 ± 10 kg/day) in mid-lactation (150 ± 50 DIM) were used. They were grouped according to parity and number of DIM and randomly assigned to one of two dietary treatments: The first group (13 cows) received a control diet (consisted of a TMR of corn and grass silages [50:50] and concentrates) supplemented with 5% SFO (SFO treatment), on DM basis, while the second group (13 cows) received the control diet supplemented with 5% LSO (LSO treatment, on DM basis) (Table 2.1) for 28 days (TP). This period of supplemental feeding was preceded by a stabilization period of 28 days during which time all cows were placed on the control diet (control period, CP). After the TP, the animals were returned to the control diet for another 28 days (PTP). The animals were housed in individual tie stalls and fed individually. They had ad libitum access to feed and water at all times. Feed intake was monitored on a daily basis by weighing the TMR offered and the refusals. The animals were weighed at the beginning and end of each feeding period.

Procedures for animal care and use were according to the national codes of practice for the care and handling of farm animals (Canadian Council on Animal Care, 2009) and approved by the animal care and ethics committee of Agriculture and Agri-Food Canada.

2.3.2 Blood collection and determination of blood metabolites

Blood samples were collected aseptically once a week from the caudal vein of each animal into ethylenediaminetetraacetic acid-coated vacutainer tubes. Samples were centrifuged at 7500 x g for 20 min at room temperature. Plasma was transferred into 2 mL tubes and stored at -20°C until analysed for the concentrations of BHBA, glucose, NEFA and TAG. Beta-hydroxybutyrate was determined by means of BHBA reagent set kit (BioPacific Diagnostic Inc. Vancouver, BC,Canada), glucose by glucose trinder assay kit (Genzyme Diagnostics, Charlottetown, PE, Canada), NEFA by Wako Chemicals (Kit HR series NEFA-HR, Richmond, VA, USA) and TAG by enzychrom TAG assay kit (Bioassay System, Hayward, CA, USA) following manufacturers' instructions.

2.3.3 Milk collection and analysis

Cows were milked twice daily (8:00 am and 6:00 pm). Equal volume of the composite sample of am and pm milking (40 mL each) was pooled and sent to a commercial laboratory (Valacta Laboratories Inc., Ste-Anne-de-Bellevue, QC, Canada) for the analysis of fat, protein, somatic cell count (SCC), urea and lactose contents. Test-day milk fat, protein, urea and lactose contents were determined using MilkoScan FT 6000 Series mid-range infrared Fourier-transform infrared-based spectrometers (Foss, Hillerod, Denmark), while milk SCC was determined by means of Fossomatic flow cytometric cell counter (Fossomatic 5000, Foss electric, Hillerod, Denmark). Daily milk yield for each cow was recorded with electronic milk meters (MU-480, De Laval Inc. Kansas City, USA).

2.3.4 Determination of the fatty acid profiles of experimental diets

Determination of fatty acid profile was carried out according to O'Fallon et al. (2007). The resulting FAME were analysed by method of GC using a Hewlett Packard 6890N gas chromatographic system (Agilent Technology, Wilmington, DE, USA) equipped with a flame ionization detector and an autosampler (Hewlett Packard, Avondale, PA, USA). The gas

chromatographic capillary column used was SLB-IL111 (100 m× 0.25 mm, 0.2 µm in thickness, Supelco, Bellefonte, PA, USA). The gas carrier used was hydrogen at 1 mL/min constant flow with the linear velocity of 26 cm/s. The column temperature was set at a start temperature of 40°C for 1 min., then ramped at 8°C /min. to 170°C and held for 1min., then 4°C /min. to 195°C and held for 2min., and finally, at 2°C /min. to 210°C and then held for 15 min. The injection port and detector temperatures were set at 250°C. The split ratio was set to 100:1 and the injection volume was 1 µl. Individual FAME peaks were identified by comparison of retention times with FAME standards (GLC No. 463 and No. UC-59-M, Nu-Chek Prep Inc., Elysian, MN, USA). C13:0 was the internal standard. Agilent Technologies Chemstation B.04.03 software was used for data analysis.

2.3.5 Statistical analysis

Statistical analyses were performed with SAS version 9.3 software (SAS Institute Inc. Cary, NC, USA). Effects of treatments on blood metabolites (glucose, TAG, BHBA and NEFA), milk components (fat, urea nitrogen, lactose, SCC and protein) and milk yield were analysed using a completely randomized design with repeated measures and mixed effects analysis of variance (ANOVA). There were more than two conditions to compare thus making the application of ANOVA F-test more powerful than a t-test (Cui and Churchill 2003). The statistical model included the fixed effects of treatment and day and random effect associated to cow,

$$Yijk = \mu + \alpha i + dij + tk + \alpha\tau ik + eijk ,$$

where Yijk, observation for animal j receiving treatment i at day k; µ, general mean; αi, fixed effect of treatment i (i = A, B); dij, random effect associated with animal j in treatment i = error term for treatment effect; τk, fixed effect of day k; ατik, interaction between treatment i and day k; eijk, random error.

2.4 Results

2.4.1 Feed intake and body weight

The feed consumption was similar for both treatments (Figure 2.1, Table 2.1). Daily feed intake decreased slightly during the TP (P <0.001) with both SFO and LSO, and PTP (P <0.05) as compared to the CP (Figure 2.1, Table 2.1). Feed intake was similar between the TP and PTP for both treatments (Figure 2.1).

Table 2.1 Daily feed intake (mean ± standard error) of cows whose diets were supplemented with 5% safflower oil or 5% linseed oil on dry matter basis.

Day	LSO treatment (kg)	SFO treatment (kg)
	Control period	
-21	52.2±2.5^{abc}	53.0±1.7^{ab}
-14	55.8±2.039^{a}	54.6±2.1^{a}
-7	55.1±1.8^{ab}	52.3±1.6^{abc}
-1	52.9±2.3^{abc}	51.4±1.6^{abcd}
	Treatment period	
+7	50.8±2.6^{abc}	49.2±1.7^{bcde}
+14	50.4±2.1^{abc}	48.6±2.0^{bcde}
+21	50.1±1.3^{bc}	49.0±1.7^{bcde}
+28	49.5±1.2^{c}	47.8±1.5^{de}
	Post treatment period	
+35	49.6±1.9^{bc}	47.3±1.2^{e}
+42	51.6±2.0^{abc}	48.9±1.5^{cde}
+49	52.1±1.7^{abc}	49.1±1.9^{bcde}
+56	53.0±1.6^{abc}	49.8±2.1^{abcde}

^{abcde}For each treatment, means within a column with different superscripts differ significantly. *(P < 0.05)

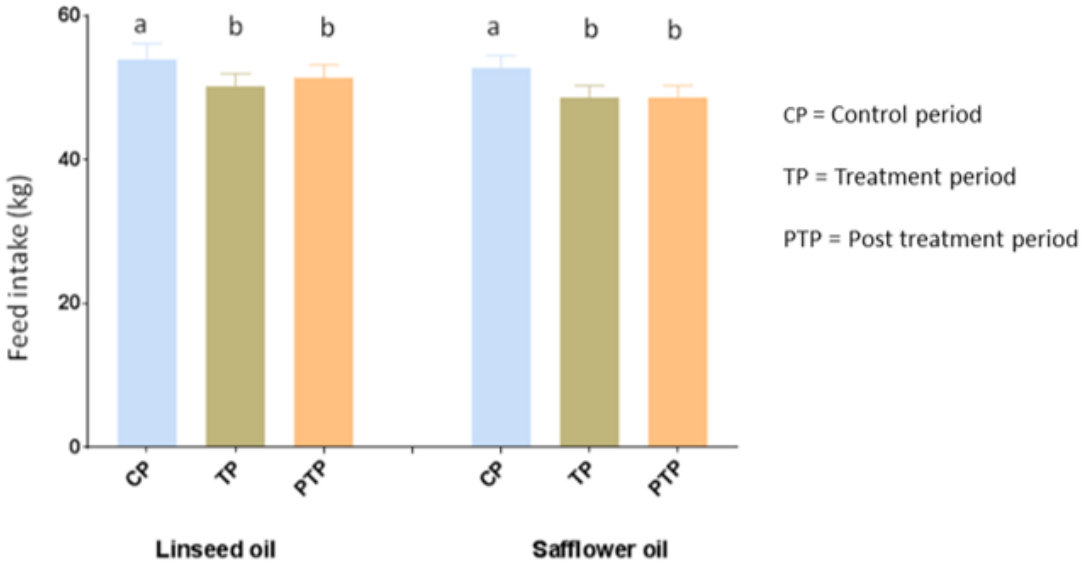

Figure 2.1 Effect of treatments on feed intake (kg) of cows during the three experimental periods (control (CP), treatment (TP) and post-treatment periods (PTP)). a, b, c = means with different letters differ significantly (P < 0.0001)

Body weight of cows increased progressively and significantly during the entire period of the experiment ($P < .0001$). For LSO treatment, BW of cows increased from 667.8 ± 25.8 to 686 ± 27.5 kg ($P < .0001$) at the end of the TP and to 710.6 ± 27.9 kg ($P < .0001$) at the end of the PTP. For SFO treatment, the increase in BW (CP = 685.4 ± 25.9 kg) at the end of the TP (696.8 ± 27.6 kg) was not significant ($P = .079$), while the increase at the end of the PTP (730.7 ± 27.1 kg) was significant ($P < .0001$) (Figure 2.2). Comparison between treatments showed similar effects of LSO and SFO on feed intake and BW gain.

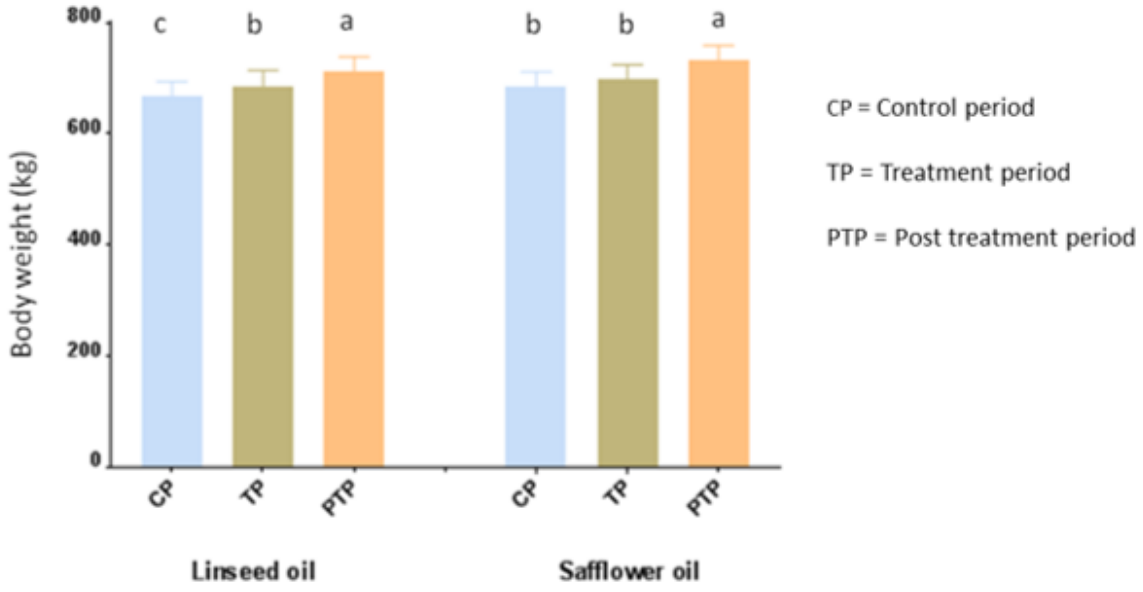

Figure 2.2 Effect of treatments on body weight (kg) of cows during the three experimental periods (control (CP), treatment (TP) and post-treatment periods (PTP)). a, b, c = means with different letters differ significantly (P < 0.0001)

2.4.2 Effect of treatments on blood metabolites

The concentrations of blood NEFA and TAG increased ($P < .0001$) during treatment with LSO and SFO as compared to the control period (Figure 2.3 and 2.4). Weekly trends show a rapid increase in NEFA and TAG concentrations for both treatments with onset of supplementation until maximum values were reached on day +28 (TP, last day of treatment) (165.05 ± 12.40 and 0.10 ± 0.01 mmol/L for LSO and 134.90 ± 11.91 and 0.10 ± 0.10 mmol/L for SFO) as compared to day −14 (89.90 ± 14.08 and 0.06 ± 0.01 mmol/L for LSO and 73.73 ± 7.28 and 0.05 ± 0.01 mmol/L for SFO), respectively (Table 2.2). With the withdrawal of treatments, there was a sharp drop in NEFA levels for both LSO and SFO until they reached control levels by day +35 (PTP) (Table 2.2). Unlike NEFA, the concentration of TAG did not return to control levels even after 28 days (day +56) of withdrawal of treatment (Figure 2.4, Table 2.2).

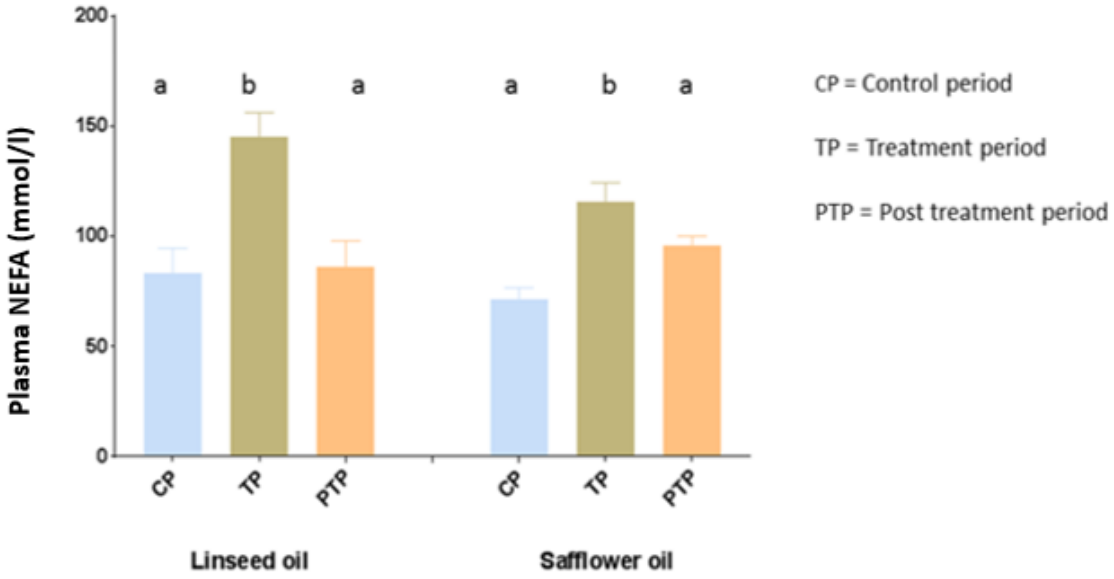

Figure 2.3 Comparison of the effect of treatments on blood NEFA during the different experimental periods (control, treatment and post-treatment periods). a, b, c = means with different letters differ significantly (P < 0.0001)

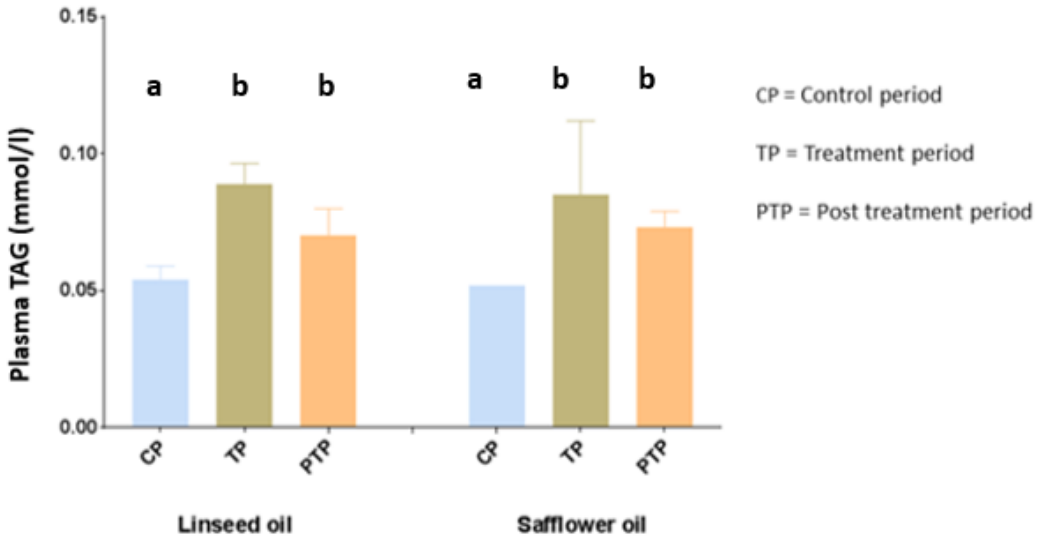

Figure 2.4 Comparison of the effect of treatments on blood TAG during the different experimental periods (control, treatment and post-treatment periods). a, b, c = means with different letters differ significantly (P < 0.0001)

Slight but significant increase ($P < .0001$) in BHBA concentration during SFO supplementation was observed but not with LSO supplementation (Figure 2.5). Furthermore, BHBA concentrations increased generally throughout the entire experimental periods and the highest values were recorded during the PTP (Figure 2.5, Table 2.2). Overall, BHBA levels continued to increase up to about four weeks after withdrawal of treatments (Figure 2.5, Table 2.2).

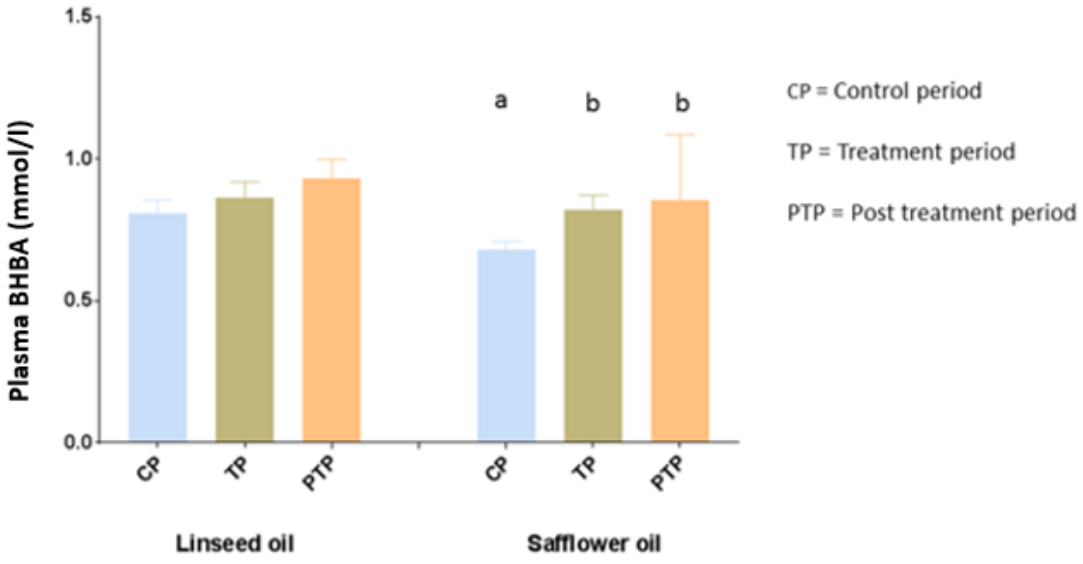

Figure 2.5 Comparison of the effect of treatments on blood BHBA during the different experimental periods (control, treatment and post-treatment periods). a, b, c = means with different letters differ significantly (P < 0.0001)

Treatment with LSO or SFO did not affect blood glucose concentrations (Figure 2.6, Table 2.2) and there were also no significant differences in blood glucose levels during the different experimental periods (CP, TP and PTP) for both treatments (Figure 2.6). Comparison between treatments showed no differences between the effects of LSO and SFO on blood NEFA, TAG, BHBA and glucose.

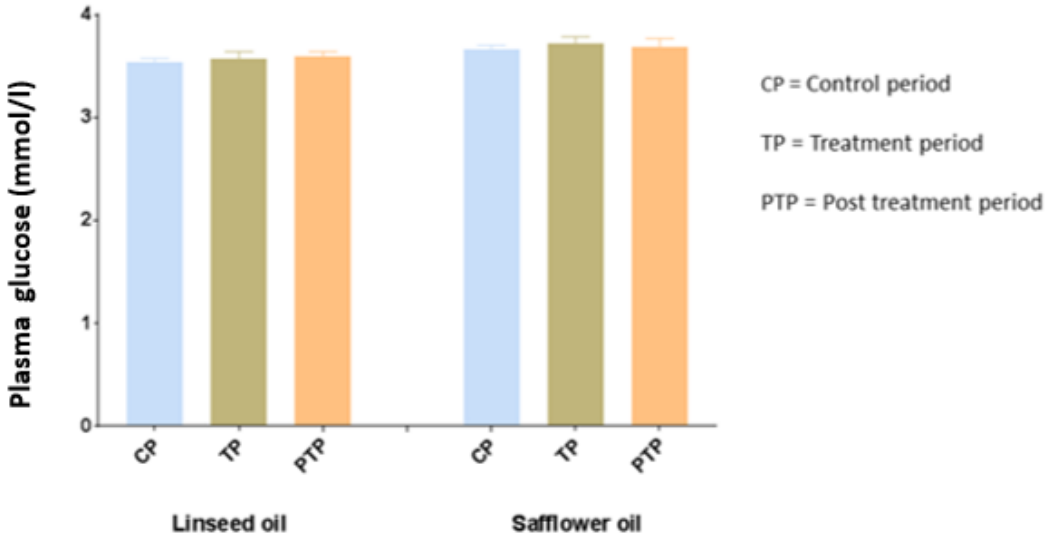

Figure 2.6 Comparison of the effect of treatments on blood glucose during the different experimental periods (control, treatment and post-treatment periods). a, b, c = means with different letters differ significantly (P < 0.0001)

Table 2.2 Day[1] effects of treatments (5% safflower oil or 5% linseed oil) on blood metabolites

Parameter	Control period		Treatment period				Post-treatment period			Overall *P*-value
	Day -14	Day -1	Day +7	Day +14	Day +21	Day +28	Day +35	Day +42	Day +56	
Safflower oil										
NEFA, mmol/L	73.73 ± 7.28	68.82 ± 3.62	96.38 ± 4.80	105.10 ± 7.57	125.70 ± 11.18	134.90 ±11.91	77.40 ± 5.76	74.60 ± 4.75	72.61 ±3.10	<.0001
TAG, mmol/L	0.054 ± 0.00	0.05 ± 0.00	0.080 ± 0.00	0.08 ± 0.00	0.08 ±0.01	0.10 ±0.10	0.08 ±0.00	0.07 ± 0.01	0.07 ± 0.01	<.0001
Glucose, mmol/L	3.69 ± 0.00	3.63 ± 0.09	3.70 ±0.09	3.64 ± 0.09	3.73 ±0.09	3.80 ±0.01	3.76 ±0.09	3.73 ± 0.10	3.55 ± 0.10	0.0654
BHBA, mmol/L	0.70 ± 0.03	0.66 ± 0.03	0.72 ± 0.05	0.88 ±0.07	0.82 ±0.03	0.89 ± 0.06	0.77 ± 0.06	0.91 ± 0.04	0.89 ± 0.06	<.0001
Linseed oil										
NEFA, mmol/L	89.10 ± 14.07	78.03 ± 7.91	124.95 ± 11.92	132.47 ± 8.12	158.06 ± 11.69	165.05 ± 12.40	94.49 ± 13.40	89.50 ± 18.45	75.03 ±3.26	<.0001
TAG, mmol/L	0.058 ± 0.00	0.05 ± 0.01	0.09 ± 0.00	0.08 ± 0.01	0.09 ± 0.01	0.098 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	<.0001
Glucose, mmol/L	3.46 ± 0.0	3.62 ±0.07	3.63 ± 0.07	3.61 ± 0.07	3.54 ± 0.07	3.53 ± 0.07	3.69 ±0.07	3.54 ± 0.07	3.55 ±0.01	0.0615
BHBA, mmol/L	0.83 ± 0.04	0.79 ± 0.05	0.79 ± 0.05	0.90 ± 0.04	0.86 ± 0.06	0.90 ± 0.08	0.87 ± 0.07	0.91 ± 0.05	1.02 ± 0.08	0.0798

[1]Values are means ± standard error.

[a-e] for each diet and parameter, row means with different superscripts differ significantly ($P < 0.05$

2.4.3 Effect of treatments on milk production and component yields

Results of milk yield (kg) during the entire period of the experiment are shown in Figure 2.7 and Table 2.3. There was a decrease in milk yield with increasing days in lactation for both treatments. Significant decrease ($P < .05$) was reached on day +28 (32.30 ± 2.94) and day +35 (31.70 ± 2.88) as compared to day −14 (36.50 ± 2.77) for LSO and on day +21 (30.29 ± 3.01), day +28 (26.91 ± 2.94), day +35 (26.05 ± 2.88), day +42 (27.70 ± 2.80) and day +56 (28.38 ± 2.81) as compared to day −14 (34.89 ± 2.77) for SFO (Table 2.3).

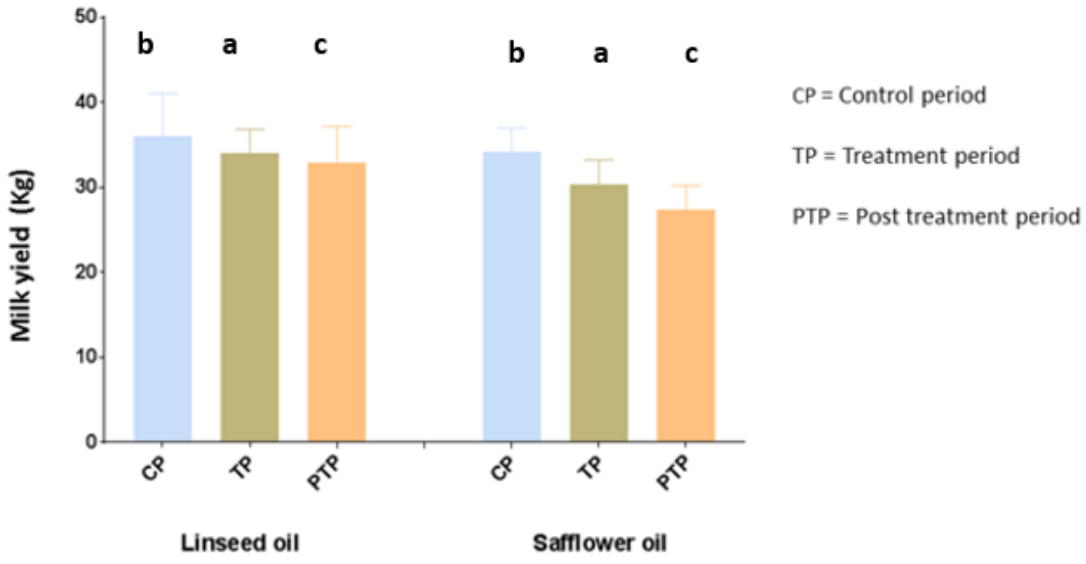

Figure 2.7 Comparison of the effect of treatments on milk yield during the different experimental periods (control, treatment and post-treatment periods)
a, b, c = means with different letters differ significantly (P < 0.0001)

Milk urea nitrogen levels decreased ($P < .0001$) during the TP as compared to CP and PTP for SFO and LSO (Figure 2.8). Weekly trends indicate that MUN levels decreased rapidly during the first week of treatment ($P < .0001$), then gradually until reaching minimum levels by day +28 ($P < .0001$) for both treatments (Table 2.3). When treatments were withdrawn, MUN levels increased rapidly, reaching control levels by day +35 for both treatments, exactly one week after cessation of treatments (Table 2.3).

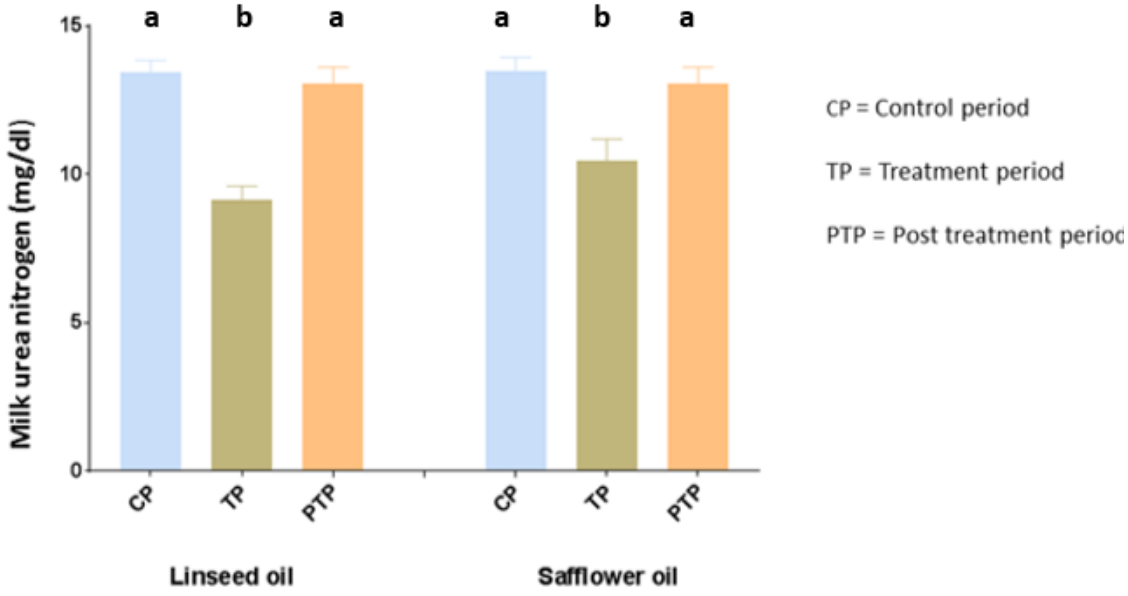

Figure 2.8 Comparison of the effect of treatments on milk urea nitrogen (MUN) during the different experimental periods (control, treatment and post-treatment periods)
a, b, c = means with different letters differ significantly (P < 0.0001)

Milk protein content increased slightly during dietary supplementation with 5% SFO but not with 5% LSO (Table 2.3). In fact, initial slight decrease for SFO or increase for LSO in milk protein content during the first week of treatment recovered and increased to levels above

control values by day +28 for SFO (3.8% ± 0.1, $P < .05$) and LSO (3.4 ± 0.1, $P = .07$) and then decreased slightly for both treatments (but never reached control levels) after one week of cessation of treatments (Table 2.3). However, no effect of treatments on milk protein% was evident when the different experimental periods were compared (Figure 2.9).

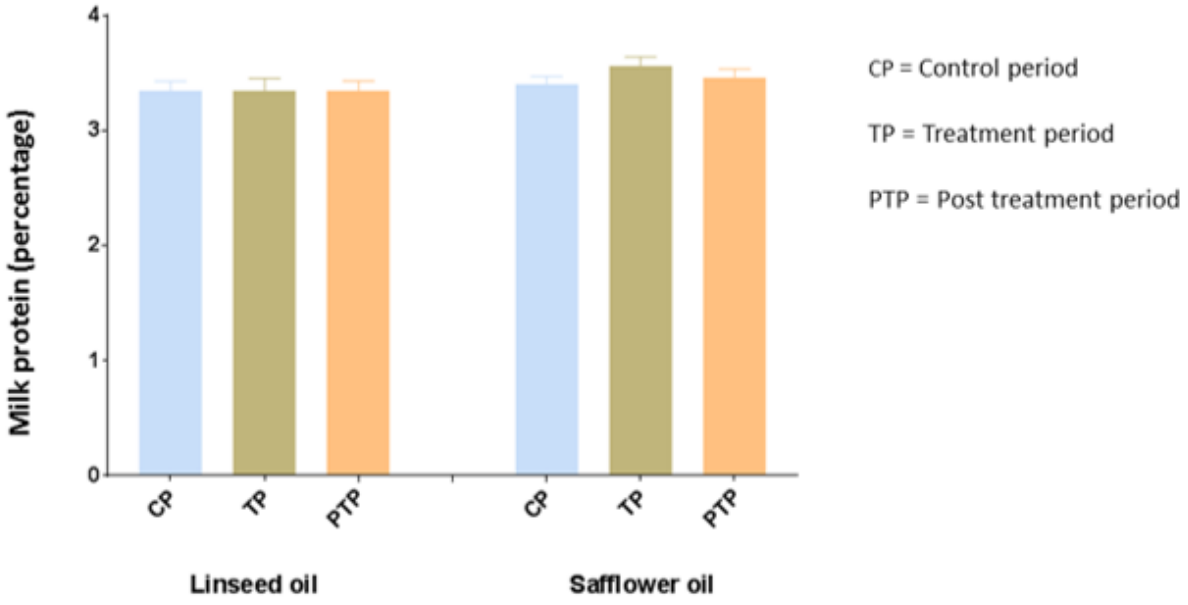

Figure 2.9 Comparison of the effect of treatments on milk protein during the different experimental periods (control, treatment and post-treatment periods)
a, b, c = means with different letters differ significantly (P < 0.0001)

Significant reduction ($P < .0001$) in milk lactose content was recorded during dietary supplementation with 5% SFO (4.30 ± 0.20%, day +28) as compared to the CP (4.60 ± 0.10%, day −14) and PTP (4.60 ± 0.10%, day +42) while no significant effect was recorded with LSO

supplementation (Figure 2.10 and Table 2.3). Treatments had no effects on milk SCC in this study when the different experimental periods were compared (Figure 2.11). Although weekly trends showed significant differences in milk SCC, there was no specific trend (Table 2.3).

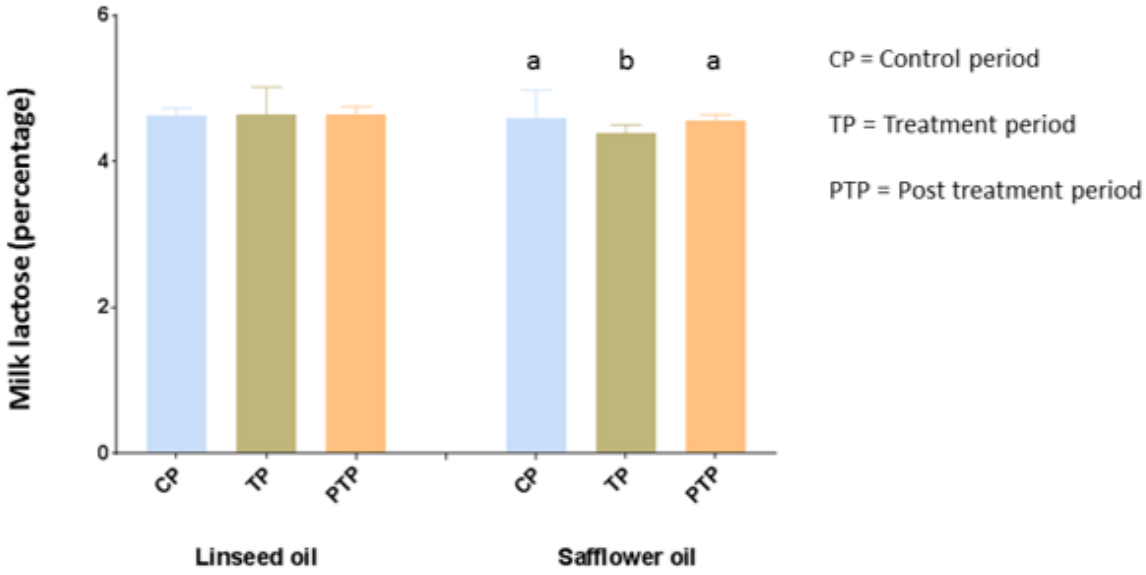

Figure 2.10 Comparison of the effect of treatments on milk lactose during the different experimental periods (control, treatment and post-treatment periods)
a, b, c = means with different letters differ significantly (P < 0.0001)

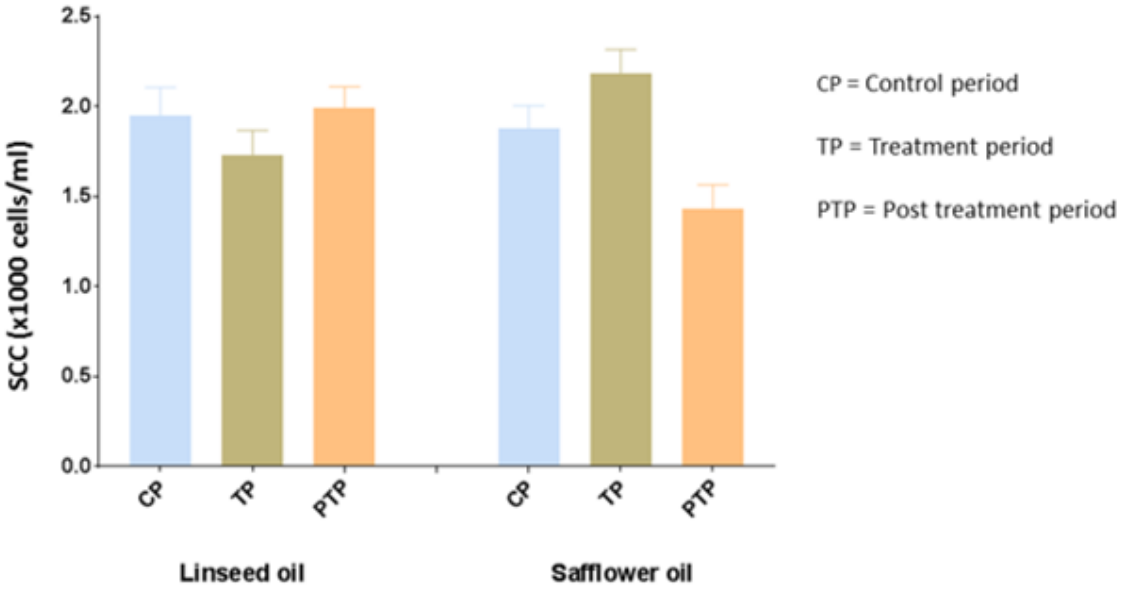

Figure 2.11 Comparison of the effect of treatments on milk SCC during the different experimental periods (control, treatment and post-treatment periods

a, b, c = means with different letters differ significantly (P < 0.0001)

Table 2.3 Day[1] effects of treatments (5% safflower oil or 5% linseed oil) on milk component yields

Parameter	Control Period		Treatment period			Post treatment period			Overall *P*-value
	Day-14	Day -1	Day+7	Day+21	Day+28	Day +35	Day+42	Day+56	
Safflower oil									
Milk yield, kg	34.89 ± 2.77	33.34 ± 2.94	33.84 ± 2.67	30.29 ± 3.01	26.91 ± 2.94	26.05 ± 2.88	27.70 ± 2.80	28.39 ± 2.81	<.0001
Fat, %	3.62 ± 0.13	3.64 ± 0.12	3.64 ± 0.12	2.45 ± 0.12	2.54 ± 0.12	3.14 ±0.13	3.51 ± 0.12	3.69 ± 0.12	<.0001
Milk urea nitrogen, mg/dl	13.20 ± 0.32	13.93 ± 0.57	10.47 ± 0.60	11.48 ± 0.65	9.48 ± 0.92	13.5 ± 0.62	13.58 ± 0.57	12.07 ± 0.51	<.0001
Protein, %	3.34 ± 0.07	3.45 ± 0.07	3.31 ± 0.06	3.61 ± 0.07	3.76 ± 0.11	3.52 ± 0.10	3.46 ± 0.07	3.39 ± 0.06	<.0001
Lactose, %	4.60 ± 0.07	4.59 ± 0.08	4.54 ± 0.07	4.34 ± 0.11	4.29 ± 0.15	4.50 ± 0.11	4.58 ± 0.11	4.58 ± 0.07	<.0001
somatic cell count, x1000 cells/ml	1.75 ± 0.12	2.01 ± 0.13	1.95 ± 0.13	2.27 ± 0.15	2.34 ± 0.13	2.10 ± 0.13	1.10 ± 0.15	1.10 ± 0.13	<.0001
Linseed oil									
Milk yield, kg	36.50 ± 2.77	35.51 ± 2.40	35.45 ± 2.67	34.10 ± 3.01	32.30 ± 2.94	31.70 ± 2.88	33.34 ± 2.80	33.74 ± 2.81	<.0001
Fat, %	3.71 ± 0.18	3.54 ± 0.17	3.73 ± 0.17	2.60 ± 0.17	2.44 ± 0.16	2.10 ± 0.17	3.48 ± 0.18	3.84 ± 0.17	<.0001
Milk urea nitrogen, mg/dl	12.49 ± 0.41	14.38 ±0.41	9.24 ± 0.50	10.28 ± 0.49	7.92 ± 0.43	13.21 ± 0.74	13.62 ± 0.46	12.35 ± 0.46	<.0001
Protein, %	3.30 ± 0.09	3.39 ± 0.09	3.31 ± 0.09	3.31 ± 0.12	3.42 ± 0.13	3.34 ± 0.10	3.38 ± 0.07	3.35 ± 0.08	<.0001
Lactose, %	4.64 ± 0.10	4.62 ± 0.10	4.63 ± 0.09	4.65 ± 0.11	4.67 ± 0.12	4.65 ± 0.13	4.65 ± 0.11	4.67 ± 0.08	0.34
somatic cell count, x1000 cells/ml	1.78±0.16	2.12±0.15	1.97± 0.14	1.10±0.14	2.13 ± 0.13	2.28 ± 0.16	1.93 ± 0.15	1.76 ± 0.10	0.0022

[1]Values are means ± standard error.

Safflower oil and LSO supplementation had similar effects on milk fat content (Figure 2.12, Table 2.3). There was a gradual decline in milk fat content from the onset of supplementation, being noticeable by day +7 and reaching minimum ($P < .0001$) levels by day +21 and day +28 for SFO and LSO, respectively, as compared to CP (day −14, middle of control peroid). When both supplements were withdrawn after 28 days of treatment, milk fat content increased gradually and reached values comparable to control levels as early as three weeks after cessation of treatments and control values at four weeks after cessation of treatments (Table 2.3). When the experimental periods were compared, dietary supplementation with LSO or SFO resulted in significant decreases ($P < .0001$) (Figure 2.12) in milk fat content during the TP as compared to the CP. Interestingly, milk fat % was significantly different ($P < .05$) between the CP and PTP, being higher during the CP (Figure 2.12). Comparison between treatments showed no differences between the effects of LSO and SFO on milk yield, MUN, protein and fat contents, lactose and SCC.

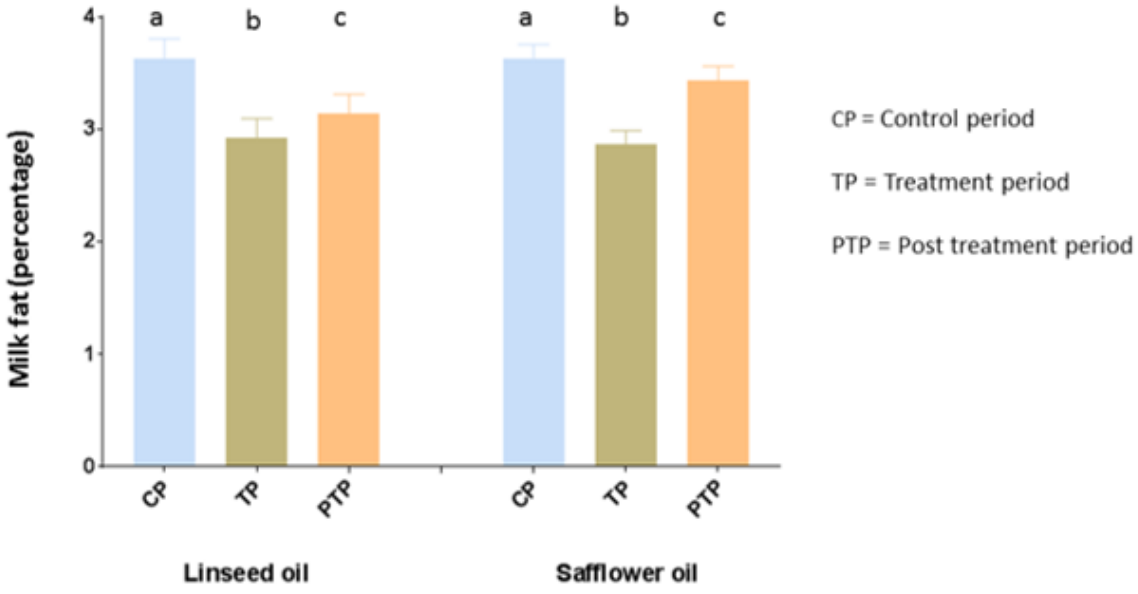

Figure 2.12 Comparison of the effect of treatments on milk fat during the different experimental periods (control, treatment and post-treatment periods)
a, b, c = means with different letters differ significantly (P < 0.0001).

2.5 Discussion

The goal of this study was to investigate the treatment and posttreatment effects of dietary supplementation of cows' diets with 5% LSO or 5% SFO on milk and blood metabolites of mid-lactation dairy cows. To measure post-treatment effects with more confidence, control, treatment and post-treatment data were obtained on the same animals to reduce bias that could arise from control animals of different genetic background. Furthermore, experimental animals were in the same stage of lactation (mid-lactation) and samples were collected within a short period of time meaning that changes in measured parameters due to a change in lactation stage was not expected to influence results.

Feeding cows with 5% LSO or SFO resulted to slight but significant reductions in feed intake during the TP in this study but this did not translate to reductions in BW. In fact, BW of animals increased progressively with time suggesting that there was enough energy from oils for animals' growth even during the period of supplemental feeding. Furthermore, reduction in feed intake especially during the first week of supplementation might be attributed to reduced palatability of the feed as a result of oil addition to the diets. Feed intake of animals, however, started to increase by the second week of supplementation but did not return to control levels even after four weeks of withdrawal of supplementation, suggesting that treatment with 5% LSO or 5% SFO might have prolonged negative effects on animal's appetite. It has been suggested previously that addition of about 10.4% of whole flaxseed to the diet of cows might reduce DM intake (Petit et al., 2002). It should be noted that the effect of USFA supplementation on DM intake vary with the amount of fat added, source of forage and the forage: concentrate ratio of the diet (Benchaar et al., 2012). In this study, milk production of cows decreased generally throughout the experimental period. Mid-lactation cows were used and consistent with the lactation curve, milk production declined linearly with increasing days in lactation. Concentrations of MUN were reduced by supplemental feeding in this study suggesting that USFA rich ingredients could greatly influence milk MUN content. This is supported by the fact that MUN levels declined rapidly and returned to control levels exactly one week after withdrawal of treatments. Our data are supported by Chichlowski et al., (2005) who reported

significantly lower concentrations of MUN and reduced (not significant) blood urea nitrogen concentrations of cows fed ground canola seeds as compared to control. Our data, however, contrast observations by several authors who found no significant effects of diets rich in USFA on MUN levels in Italian Friesian (Marchesini et al., 2009) and Brown Swiss cows (Sigl et al., 2010). The concentration of urea in milk is monitored in many dairy herds as an indicator of the efficiency of protein utilization (Rafieei 2011). Milk urea nitrogen value can be elevated when excess rumen degradable protein is fed to the cows or when rumen degradable protein is not balanced with the dietary non-fibre carbohydrates. When this happens, the unutilized portion of the dietary crude protein is converted to urea by the liver and is circulates into milk, blood and urine (Powell et al. 2014).

Milk protein content was increased slightly (for SFO by day +28) by dietary supplements in this study. Our result is supported by several authors who reported an increase (Fuentes et al., 2008; Petit et al., 2002; Mach et al., 2011) but contradicts reports that recorded a decrease (Ward et al., 2002) or who found no effect (Dhiman et al., 2000; Huang et al., 2008) of supplemental feeding of a variety of USFA ingredients to dairy cows on milk protein content. Milk SCC was not affected by treatments in this study. Animals were well cared for and farm management ensures excellent practices that reduced infection rates during the entire period of the experiment. The use of SCC to monitor possible infections is normally meant to detect "clinical mastitis", which dramatically increases SCC counts. However, SCC counts are not precise enough to be sure that there is no "subclinical infections" where the bacteria are present and affect the mammary gland integrity and metabolism.
In this study, milk lactose content was reduced significantly with dietary supplementation with SFO but not with LSO. Our data on LSO are supported by several authors who observed no effect of feeding USFA on milk lactose concentration (Martin et al., 2008; Secchiari et al., 2003; Dai et al., 2011).

As expected, milk fat content decreased by 34.2% and 29.9% with 5% LSO and 5% SFO supplementation, respectively. This result is consistent with reports by other authors (Bell et al., 2006; Murphy et al., 2008; Angulo et al., 2012) who recorded reductions of up to 50% in milk

fat content following supplementation of dairy cow diets with a variety of materials rich in USFA, generally referred to as MFD. Dietary lipids are extensively altered by bacterial metabolism in the rumen, and one of the major changes is the biohydrogenation of PUFA (Bauman and Griinari 2003). During this process, the rumen environment alters the pathways of biohydrogenation and increased rumen outflow of *trans*-isomer like *trans*-10, *cis*-12-CLA which is one intermediate shown unequivocally to inhibit milk fat synthesis (Bauman and Griinari 2003; Bauman et al.2011). However, increases in ruminal *trans*-10, *cis*-12-CLA formation do not provide a universal justification for the decline in milk fat during diet-induced MFD, signifying that other biohydrogenation intermediates may also be involved (Shingfield and Griinari 2007). The supplemental ingredients used in this study were rich in ALA (LSO) or LA (SFO) and caused reductions of about 34.2% and 29.9% in milk fat content, respectively, thus supporting the notion that other intermediates of biohydrogenation other than *trans*-10, *cis*-12-CLA were also involved. Furthermore, we analysed the fatty acid content of a subset of the same animals (12) and observed significant increase in the contents of *trans*-10, *cis*-12-CLA by treatments (Ibeagha-Awemu et al., 2016) thus supporting its involvement in the process of depressing milk fat production in this study. Moreover, gene expression data showed a down-regulation of genes involved in mammary lipogenesis or milk fat synthesis (Ibeagha-Awemu et al., 2016) and supported by several studies in which cows were fed milk fat-depressing diets (Ahnadi et al., 2002; Harvatine and Bauman 2006; Angulo et al., 2012). Interestingly, the factors that were responsible for the inhibitory effect on milk fat synthesis in this study continued to have an effect up to three weeks after withdrawal of supplemental feeding, suggesting that the fatty acid intermediates were not immediately cleared from the system after removal of the dietary supplements. This may account for the significant low milk fat content in the PTP as compared to the CP in this study. To fully validate the effect of USFA on milk fat reduction, a control that consist of dietary supplementation of 5% SFA would have been necessary.

Increased levels of TAG during dietary supplementation with 5% LSO or 5%SFO in this study may be a result of increased availability of ruminal biohydrogenation products of PUFA (Ibeagha-Awemu et al., 2016) which were absorbed or eventually transported and stored in the adipose tissues and liver as TAG. Our findings are in agreement with Vafa et al., (2010) who reported a similar increase in TAG concentration in serum when cows in early lactation received

increasing levels of dietary fatty acid from canola seeds (1%canola oil + 1% fish oil or 2% canola oil) but contradicts Alizadeh et al., (2010) who found no influence of dietary supplementation of different combinations of cotton and safflower seeds on blood TAG of Holstein cows in the early stage of lactation. It has been demonstrated that supplemental fat can increase the blood NEFA concentration of dairy cows during the transition period and early lactation (Rennó et al., 2014). Blood NEFA returned to control levels by day +35, exactly one week after the withdrawal of treatment thus suggesting that increased NEFA levels in the blood of treated animals were from increased availability of ruminal biohydrogenation products of PUFAs. However, TAG levels never returned to control levels even after 28 days of withdrawal of treatment suggesting that the conversion process of NEFA to TAG and its transportation as low-density lipoprotein from blood to the liver may be slower than its breakdown. Vast amount of NEFA is metabolized in the liver through three main steps or pathways; the first step involves complete oxidation leading to production of water, adenosine triphosphate and carbon dioxide, the second step involves incomplete oxidation leading to the production of ketones, while the third step results to esterification of TAG (Li et al., 2013). High blood NEFA content can be incompletely oxidized to ketones (BHBA) or esterified to TAG leading to ketosis (fatty liver syndrome) (Adewuyi et al., 2005) or secreted in milk by the udder (Drackley 1999).

Our results on BHBA (Figure 2.2 C, Table 2.2) are in line with Mendoza et al. (2008) who noted that dietary supplementation with whole sunflower seeds (rich in PUFA) tended to increase BHBA concentrations in grazing dairy cows. However, Alizadeh et al., (2010) reported a linear decline in blood BHBA when cows at the early stage of lactation were fed diets supplemented with safflower seeds (1.4 kg per cow per day). Moussavi et al., (2007) also observed no significant effect of supplemental feeding of 5% fish meal and n-3 fatty acids to early lactating dairy cows on BHBA.

Generally, treatments did not affect blood glucose concentrations in this study (Figure 2.2 D and Table 2.2) which is consistent with reports by other authors (Rafalowski and Park 1982; Sigl et al., 2010).

In this experiment, the design never took into consideration the effect of time to ensure that the observed effects of diet are not simply due to time, especially the drop in milk production which drops with time. Additionally, comparison between different animals and using the differences as covariates in the statistical analysis. With all these into consideration, a new design for future studies should include two more groups, one control group having no treatment to account for differences that can occur in time, one group fed with 5% SFA to account for differences due to USFA in the diet. Also, a better monitoring of possible subclinical infection would be important in future studies. The latter could be done by bacteriology tests on each quarter throughout the monitoring period. Finally, dietary supplementation with 4% LSO or SFO instead of 5% to maintain feed intake.

2.6 Conclusion

Dietary supplementation of cows' diets with 5% SFO or 5% LSO influenced blood metabolites and milk components during the treatment and post-treatment periods. Unlike NEFA, the concentration of TAG did not return to control levels even after 28 days (day+56) of withdrawal of treatment. Overall, BHBA levels continued to increase after withdrawal of treatments, reaching significant levels by day +49 (three weeks after cessation of treatment). Milk fat content decreased by 34.2% (LSO) or 29.9% (SFO) during treatments and its concentrations returned to control levels after three weeks of cessation of treatments. Milk protein content increased slightly during treatment (significant for SFO by day +28) and decreased to control levels one week after cessation of treatments. MUN was decreased by both treatments and levels increased rapidly following withdrawal of treatments reaching control levels by day +35, exactly one week after cessation of treatments. Our data shows that the residual effects of feeding USFA on the physiology of dairy cows were still active up to three weeks after cessation of treatments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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CHAPTER 3

miRNA clusters and miRNA-mRNA pairs potentially regulating milk traits and blood metabolites

Article Description

In this manuscript, we described the possible molecular mechanisms involved in the regulation of blood and milk metabolites in the bovine mammary gland following dietary supplementation with unsaturated fatty acids (USFA). Transcriptome analysis of mammary gland tissues of the cows following dietary supplementation with USFA identified differentially expressed genes (mRNAs and miRNAs) with a greater impact of LSO as compared to SFO on studied traits (Ibeagha-Awemu et al. 2016, Li et al. 2015). Genes usually act in concert to influence the phenotypic expression of a trait. Furthermore, miRNAs are known to regulate the activities of over 60% of protein coding genes and of almost all biological processes (Bose and Bose, 2016). Therefore, we studied the co-expression patterns between miRNAs, and miRNA-mRNA using transcriptome data of the same animals (Ibeagha-Awemu et al., 2016; Li et al., 2015) and their influence on blood and milk metabolites. We used the weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) R-package to detect modules or clusters of miRNAs that are similarly expressed and miRNA–mRNA co-expressed pairs in the mammary gland following dietary supplementation with USFA and the relationships between miRNA modules/miRNA-mRNA pairs and studied traits (blood and milk metabolites). We identified three consensus modules (blue, brown and turquoise) from control and treatment with LSO or SFO. Furthermore, 68, 6 and 2 miRNA-mRNA pairs were found to be significantly correlated with at least one phenotype in the blue, brown and turquoise modules, respectively.

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Author Contributions

Conception and design of the study: Eveline Ibeagha-Awemu; Provided inputs on study design: Nathalie Bissonnette and Nicolas Gévry. Data collection: Adolf Ammah; Data analysis: Adolf Ammah, Do Duy; Interpretation of data: Adolf Ammah, Do Duy, Eveline Ibeagha-Awemu; Drafting of manuscript: Adolf Ammah and Do Duy; Critical revision of the manuscript: Eveline Ibeagha-Awemu; Revised and approved the final manuscript: All authors.

Co-Expression Network Analysis Identifies miRNA–mRNA Networks Potentially Regulating Milk Traits and Blood Metabolites

Adolf A. Ammah [1, 2], Duy N. Do[1, 3], Nathalie Bissonnette [1], Nicolas Gévry[2], and Eveline M, Ibeagha-Awemu [1*]

[1] Agriculture and Agri-Food Canada, Sherbrooke Research and Development Centre, 2000 College Street, Sherbrooke, QC J1M 0C8, Canada

[2] Department of Biology, University of Sherbrooke, 2500 University Boulevard, Sherbrooke, QC J1K 2R1, Canada

[3] Department of Animal Science, McGill University, 21111 Lakeshore Road, Ste. Anne de Bellevue, QC H9X 3V9, Canada

***Correspondance: Eveline.ibeagha-awemu@agr.gc.ca**

3.1 Abstract

MicroRNAs (miRNA) regulate mRNA networks to coordinate cellular functions. In this study, we constructed gene co-expression networks to detect miRNA modules (clusters of miRNAs with similar expression patterns) and miRNA–mRNA pairs associated with blood (TAG and NEFA) and milk (milk yield, fat, protein, and lactose) components and milk fatty acid traits following dietary supplementation of cows' diets with 5% LSO (n = 6 cows) or 5% SFO (n = 6 cows) for 28 days. Using miRNA transcriptome data from mammary tissues of cows for co-expression network analysis, we identified three consensus modules (common function/processes): blue, brown, and turquoise, composed of 70, 34, and 86 miRNA members, respectively. The hub miRNAs (miRNAs with the most connections with other miRNAs) were miR-30d, miR-484 and miR-16b for blue, brown, and turquoise modules, respectively. Cell cycle arrest, and p53 signaling and transforming growth factor–beta (TGF-β) signaling pathways were the common GO and KEGG pathways enriched for target genes of the three modules. Protein percent (P = 0.03) correlated with the turquoise module in LSO treatment while protein yield (P = 0.003) and milk yield ($P = 7 \times 10^{-04}$) correlated with the turquoise model, protein and milk yields and lactose percent ($P < 0.05$) correlated with the blue module and fat percent (P = 0.04) correlated with the brown module in SFO treatment. Several fatty acids correlated ($P < 0.05$) with the blue (CLA:9,11) and brown (C4:0, C12:0, C22:0, C18:1n9c and CLA:10,12) modules in LSO treatment and with the turquoise (C14:0, C18:3n3 and CLA:9,11), blue (C14:0 and C23:0) and brown (C6:0, C16:0, C22:0, C22:6n3 and CLA:10,12) modules in SFO treatment. Correlation of miRNA and mRNA data from the same animals identified the following miRNA–mRNA pairs: miR-183/*RHBDD2* (P = 0.003), miR-484/*EIF1AD* (P = 0.011) and miR-130a/*SBSPON* (P = 0.004) with lowest P -values for the blue, brown, and turquoise modules, respectively. Milk yield, protein yield, and protein percentage correlated ($P < 0.05$) with 28, 31 and 5 miRNA–mRNA pairs, respectively. Our results suggest that, the blue, brown, and turquoise modules miRNAs, hub miRNAs, miRNA–mRNA networks, cell cycle arrest GO term, p53 signaling and TGF-β signaling pathways have considerable influence on milk and blood phenotypes following dietary supplementation of dairy cows' diets with 5% LSO or 5% SFO.

Keywords: co-expression; microRNA; mRNA, transcription factor; blood metabolites; milk components; milk fatty acids

3.1 Résumé

Les microARNs (miARNs) régularisent les réseaux d'ARNm pour coordonner les fonctions cellulaires. Dans cette étude, nous avons construit des réseaux de co-expression de gènes pour détecter les modules de miARN (grappes de miARN avec des modèles d'expression similaires) et les paires de miARN associés au sang (triacylglycérides et acides gras non estérifiés) et au lait (rendement laitier, matières grasses, protéines et lactose) et les caractères des acides gras du lait après supplémentation alimentaire des régimes de vaches avec 5% d'huile de lin (LSO) (n = 6 vaches) ou 5% d'huile de carthame (SFO) (n = 6 vaches) pendant 28 jours. En utilisant les données du transcriptome miRNA des tissus mammaires des vaches pour l'analyse du réseau de co-expression, nous avons identifié trois modules de consensus: bleu, brun et turquoise, composés respectivement de 70, 34 et 86 membres miRNA. Les miRNAs de moyeu (miRNAs avec le plus grand nombre de connexions avec d'autres miRNAs) étaient miR-30d, miR-484 et miR-16b pour les modules bleu, brun et turquoise, respectivement. L'arrêt du cycle cellulaire et les voies de signalisation et de transformation du facteur de croissance-bêta (TGF-β) étaient l'ontologie commune des gènes (GO) et les voies de l'Encyclopédie des gènes et des génomes (KEGG) de Kyoto enrichies pour les gènes cibles des trois modules. Le pourcentage de protéines (P = 0,03) est corrélé avec le module turquoise dans le traitement LSO tandis que le rendement en protéines (P = 0,003) et le rendement en lait (P = 7 × 10-04) sont corrélés avec le modèle turquoise, les rendements en protéines et en lait et le pourcentage de lactose (P < 0,05) sont corrélés avec le module bleu et le pourcentage de matières grasses (P = 0,04) est corrélé avec le module brun dans le traitement SFO. Plusieurs acides gras corrélés (P < 0.05) avec les modules bleus (CLA:9,11) et bruns (C4:0, C12:0, C22:0, C18:1n9c et CLA:10,12) en traitement LSO et avec les modules turquoise (C14:0, C18:3n3 et CLA:9,11), bleu (C14:0 et C23:0) et brun (C6:0, C16:0, C22:0, C22:6n3 et CLA:10,12) en traitement SFO. La corrélation des données miRNA et mRNA des mêmes animaux a permis d'identifier les paires miRNA-mRNA-mRNA suivantes: miR-183/RHBDDD2 (P = 0,003), miR-484/EIF1AD (P = 0,011) et miR-130a/SBSPON (P = 0,004) avec les valeurs p les plus faibles pour les modules bleu, brun et turquoise, respectivement. Rendement en lait, rendement en protéines et pourcentage de protéines corrélés (P < 0,05) avec 28, 31 et 5 paires miARN-ARNm-ARNm respectivement. Nos résultats

suggèrent que les modules bleu, brun et turquoise miRNAs, miRNAs hub, miRNAs, réseaux miRNA-mRNAs, arrêt du cycle cellulaire GO terme, p53 signalisation et TGF-β voies de signalisation ont une influence considérable sur le lait et les phénotypes sanguins suite à la supplémentation alimentaire des régimes des vaches laitières avec 5% LSO ou 5% SFO.

Mots-clés: co-expression; miARN; ARNm, facteur de transcription; métabolites sanguins; composants du lait; acides gras du lait.

3.2 Introduction

Bovine milk and its products constitute a rich source of proteins, energy, minerals (e.g., calcium), vitamins (A, B, D, E and K) and antioxidants in human nutrition. Milk supplies unsaturated fatty acids (USFA) which have been associated with decreased risk of cardiovascular diseases (stroke, high blood pressure, heart failure and coronary heart diseases), inflammatory diseases and some types of cancers (Kris-Etherton and Innis, 2007; Griel and Kris-Etherton, 2006; Parodi, 2005). Unsaturated fatty acids make up about 30% of the fatty acid content of milk, meanwhile it has been proposed that milk fat composition with potential positive effects on human health should contain about 70% USFA (Soyeurt et al., 2006). Nutrition is one of the factors that greatly impacts milk fat composition and the largest changes in milk fatty acid composition have been obtained either by changing the amounts and the nature of forages in the diets of cows, particularly pasture, or by adding plant or marine oils to the diet (Chilliard et al., 2007; Dewhurst et al., 2006). Plant products like linseed, soybeans, safflower and sunflower are the most effective sources of unsaturated plant lipids used to enhance the CLA and USFA contents of milk fat. Unsaturated fatty acids and other factors like physiological and metabolic state of the cow, breed and genetics are known to influence the concentration of blood metabolites like glucose, NEFA, TAG and BHBA (Ammah et al., 2018; Rennó et al., 2014) For instance, we reported significant increases in blood NEFA and TAG concentrations and significant reductions in milk fat and MUN contents in Holstein cows following dietary supplementation with USFA (Rennó et al., 2014). Moreover, the blood metabolic profile of dairy cows is used to assess the nutritional and health state of the dairy herd (Hashemzadeh-Cigari et al., 2015; Lee et al., 1978).

In our previous transcriptome studies of the bovine mammary gland, we identified mRNAs and miRNAs that were differentially expressed in response to diets rich in USFA (Ibeagha-Awemu et al., 2016; Li et al., 2015) . Furthermore, we also examined the effect of diets rich in USFA on milk composition (fat, protein, milk yield and lactose) and blood metabolites (TAG and NEFA) of lactating Holstein cows (Ammah et al., 2018). The mRNA transcriptome analysis identified 1006 (460 up and 546 downregulated) and 199 (127 up and 72 down-regulated) genes that were significantly differentially regulated after LSO and SFO

supplementation, respectively, meanwhile the miRNA transcriptome analysis detected 14 and 22 miRNAs significantly differentially regulated by LSO and SFO, respectively. Since a network of genes and regulatory factors work in concert to influence the phenotypic expression of traits, assessment of gene expression without taking into account the factors that regulate their activities may not adequately explain the complex biological mechanisms underlying the expression of traits. Micro ribonucleic acids interact with mRNA(s) to regulate their expression and consequently biological processes, so it is important to study their synergistic effects on the phenotypic expression of traits. Hence, an integrative approach in assessing gene expression in a network basis is necessary to unravel the molecular mechanism underlying milk fat traits.

Network approaches have proven to be powerful tools for exploring the biological mechanisms underlying complex traits (Kogelman et al., 2014; Cho et al., 2012; Weiss et al., 2012). It has been widely applied on output data from high throughput sequencing technology to identify key regulators and pathways in human diseases such as cancer and obesity (Wang et al., 2009), type 1 diabetes (Riquelme and Lubovac-Pilav, 2016), Alzheimer's disease (Seyfried et al., 2017), livestock production traits (Kogelman et al., 2014; Kogelman et al., 2014; Kogelman et al., 2015; Suravajhala et al., 2016) and functional annotation of cattle genes (Beiki et al., 2016). Moreover, understanding gene networks also helps to better guide genomic selection in animal breeding programs (Snelling et al., 2013).

In order to understand gene interaction, different methods have been developed to construct co-expression networks and to identify modules of highly connected genes. The weighted gene co-expression network analysis (WGCNA) is among the most established and widely used of such methods (Langfelder and Horvath, 2008). We and other authors have successfully used WGCNA to identify key genes and networks for various complex traits in livestock species such as meat quality traits in pigs (Ponsuksili et al.,2013) feed efficiency in cattle (Alexandre et al., 2015) and milk yield and component traits in cows (Do et al., 2017). Furthermore, integrative omics approaches have been applied on combined mRNA and miRNA expression data to detect major regulatory mechanisms in different phenotypes such

as carcass and meat quality traits in porcine (Ponsuksili et al., 2013), abnormality in breast cancer patients (Lee and Jiang, 2017) and colorectal cancer (Cantini et al., 2015). Moreover, the consensus module approach (finding common functions/processes) has proven to be a promising method for finding hub genes and regulators across different datasets (Horvath et al., 2012; Iancu et al., 2013a; Huang et al., 2014; Langfelder et al., 2013 Iancu et al., 2013b; Miller et al., 2014). Such hub genes and regulators may form targets for further functional validation of their roles in identified networks. Furthermore, the identification of key miRNAs, their networks, and their downstream target genes and pathways might also facilitate the use of genetic engineering technologies, such as RNA interference technologies or gene editing, to obtain desired phenotypes by controlling the expression of miRNAs and/or their target genes. Moreover, the miRNAs, genes, and network information might be useful for genomic predictions (Kadarmideen, 2014; Gao et al., 2015). However, these approaches have not yet been applied to explore the regulatory mechanisms in the bovine mammary gland in response to diets rich in USFA (SFO or LSO). Therefore, this study aimed to (i) construct consensus modules across miRNA expression data from control, LSO and SFO treatments using the WGCNA approach; (ii) correlate important miRNA modules with milk and blood component phenotypes; (iii) enrich target genes (mRNA) of miRNAs from important modules to explore the possible biological processes, pathways and transcriptional regulators regulating milk and blood component phenotypes and (iv) identify miRNA–mRNA networks regulating milk and blood component phenotypes.

3.3 Materials and Methods

3.3.1 Animal Management and Sampling

Animal management and sampling procedures were according to the national codes of practice for the care and handling of dairy cows (http://www.nfacc.ca/codes-of-practice) and approved by the Animal Care and Ethics Committee of Agriculture and Agri-Food Canada (CIPA#402, 04 April 2012).

Detailed procedures for animal management and data collection have been reported previously (Li et al., 2015a). Briefly, 12 Canadian Holstein cows in mid-lactation were randomly assigned to either LSO or SFO treatment (6 cows/treatment). These animals were fed a control diet of TMR of corn and grass silages (50:50) and concentrates for 28 days (CP), after which the control diet was supplemented with 5% LSO or 5% SFO on a DM basis (treatment period) for another 28 days. Animals were milked every day at 8:00 am and 6:00 pm. Analysis of fat, protein and lactose contents in milk samples collected on days −14 and −1 (CP) and +7, +14, +21 and +28 (TP) were done using 80 mL of milk (pool of morning (40 mL) and evening (40 mL) milk) by a commercial laboratory (Valacta Laboratories Inc., Ste. Anne de Bellevue, QC, Canada). Daily milk yield for each cow was recorded with electronic milk meters (MU-480, De Laval Inc., Kansas City, MO, USA). Milk fat from milk samples (40 mL) was extracted by centrifugation at 4500 x *g* for 20 min at 4 °C.

Blood samples were collected aseptically in an ethylenediaminetetraacetic acid vacutainer tube, from animals on days −14 and −1 (CP) and +7, +14, +21 and +28 (TP) and centrifuged at 7500 x *g* for 20 min at room temperature. The resulting plasma was used for the analysis of NEFA (Wako Chemicals, Kit HR series NEFA-HR, Richmond, VA, USA) and TAG (enzychrom TAG assay kit, Bioassay System, Hayward, CA, USA), following manufacturers' instructions.

Mammary biopsies were collected from animals in each group on day −14, day +7 and day +28 which corresponded to middle of CP, early treatment and end of treatment periods, respectively, following an established protocol (Farr et al., 1996). Biopsies were snap frozen in liquid nitrogen and stored at −80 °C pending isolation of total RNA.

3.3.2 **RNA Isolation**

Procedures for RNA isolation have been reported previously (Ibeagha-Awemu et al., 2016). In brief, 50 mg of mammary gland biopsy sample was used for total RNA isolation using miRNeasy Kit (Qiagen Inc., Toronto, ON, Canada) following manufacturer's

instructions. The Turbo DNase Kit (Ambion Inc., Foster City, CA, USA) was used to remove contaminating DNA from isolated RNA. The Nanodrop ND-1000 instrument (NanoDrop Technologies, Wilmington, DE, USA) was used to measure RNA concentration and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to accessed RNA quality. The RNA Integrity Numbers of all the samples were > 8.

3.3.3 mRNA Sequencing and Data Processing

Procedures for mRNA sequencing and data processing have been reported previously (Ibeagha-Awemu et al., 2016). Briefly, 250 ng of total RNA/sample was use for library generation using TruSeq stranded mRNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA). The Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Burlington, ON, Canada) and the Kapa Illumina GA with the Revised Primers-SYBR Fast Universal Kit (D-Mark Biosciences, Toronto, ON, Canada) were used to quatify prepared libraries. The 2100 Bioanalyzer instrument (Agilent Technologies) was used to determine the average fragment size of libraries. Generated libraries (36) were multiplexed and subjected to 100 bp paired-end sequencing on six lanes of a HiSeq 2000 system (Illumina Inc.) by McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada). Generated reads were processed using a pipeline developed by McGill University and Genome Quebec Innovation Centre (http://gqinnovationcenter.com/).

3.3.4 miRNA Sequencing and Data Processing

Procedures for miRNA library preparing, sequencing and bioinformatics management of data have been reported previously (Li et al., 2015)**.** Briefly, the the procedure for miRNA library preparation and barcoding for sequencing was according to Vigneault et al. (2012) with slight modifications (Li et al., 2015). Total RNA was first ligated to a primer (adaptor) at the 3′ by T4 RNA Ligase 22tr K227Q (New England Biolabs Inc., Canada) and 5′ ends by T4 RNA Ligase 1 (Enzymatics Inc., a division of Qiagene Inc., Beverly, MA USA) followed

by reverse transcription into *cDNA* using Superscript III Kit (Life Technologies, Carlsbad, CA, USA). Barcoding of the different libraries was done followed by size separation by polyacrylamide gel electrophoresis and finally the concentration of the purified libraries was assessed by PicoGreen assay (Life Technologies, USA) on a Nanodrop 3300 fluorescent spectrophotometer. Multiplexed libraries were sequenced on 3 lanes on an Illumina HiSeq 2000 system (Illumina Inc., USA) by McGill University and Genome Quebec Innovation Centre (Montreal, QC, Canada).

The FastQC program version 0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to check sequencing quality. Then, the cutadapt v1.2.2 program (http://code.google.com/p/cutadapt/) was used to trim adaptor sequences. Clean reads were parsed into one and mapped to the bovine genome (Bta_4.6.1) using bowtie 1.0.0. (Langmead et al., 2009). Reads that mapped to 1 to 5 positions were further used (mismatched reads). miRDeep2 v2.0.0.5 tool was used to identify known miRNAs and also in the discovery of novel miRNAs.

3.3.5 Fatty Acid Analysis

Fatty acid methyl ester preparation and quantification of fatty acid profiles have been reported previously (Ibeagha-Awemu et al., 2016). Briefly, fatty acid methyl esters were prepared and quantified using the Hewlett Packard 6890 N gas chromatographic system (Agilent Technology, Wilmington, DE, USA). The carrier gas was hydrogen and the capillary column used was SLB-IL111 (100 m × 0.25 mm, 0.2 µm in thickness, Supelco, Bellefonte, PA, USA). The column temperature was set at a start temperature of 40°C for 1 min., then ramped at 8°C /min. to 170°C and held for 1min., then 4°C /min. to 195°C and held for 2min., and finally, at 2°C /min. to 210°C and then held for 15 min. Individual fatty acids were determined by comparing their retention time with that of fatty acid methyl esters standards (GLC No. 463 and No. UC-59-M, Nu-Chek Prep Inc., Elysian, MN, USA). The Chemstation B.04.03 software (Agilent technologies) was used for data analysis.

3.3.6 Construction of Gene Co-Expression Networks

The expression of miRNAs was normalized using Deseq2 package (v1.11.19) (Love et al., 2014) and the final normalized matrix of 321 miRNAs were used as input for co-expression network analysis using the WGCNA R-package (Langfelder and Horvath, 2008). For WGCNA analyses, a signed co-expression measure for each pair of miRNAs was computed based on their co-expression level. Then weighted adjacency matrix was calculated from the signed co-expression measure using a power function. A topological overlap measure (TOM) was then calculated based on a combination of a value between the adjacency of two miRNAs and the connection strength that the two miRNAs share with other miRNAs. A TOM of 0 or 1 was assigned to each pair of miRNAs. When miRNAs shared the same neighbor, a TOM value of 1 is assigned while a TOM value of 0 indicates that they do not share any neighbor. To produce a clustering tree (dendrogram), the dynamic tree−cutting algorithm was used (Langfelder et al., 2008). To construct the consensus module, the blockwiseConsensusModulesfunction was run (Langfelder and Horvath, 2007; Langfelder and Horvath, 2014) with option of soft-thresholding power for network construction of 9, and minimum module size of 20. Moreover, the medium threshold was also applied to control the sensitivity of module detection (deepSplit of 2) and to merge modules in the dynamic tree (mergeCutHeight of 0.25). The signed network option was chosen when constructing the consensus modules. In the gene network, a gene might interact with many others to perform its function, therefore, a minimum module size of 30 genes has been recommended for the gene network construction, by the software developers (Langfelder and Horvath, 2008). Since a lower number of miRNAs might interact with each other to form networks (Xu et al.,2010; Xiao et al., 2012) we applied a lower threshold of 20 miRNAs for minimum module size. Each branch of a tree is a module and a module with at least 20 miRNAs was assigned to a color (Figure S1). Details about WGCNA and its merits have been reported previously (Zhang and Horvath, 2005; Langfelder and Horvath, 2007, 2008).

3.3.7 Module−Trait Relationship

Module−trait relationships were computed based on Pearson's correlation between the module eigengene and blood and milk components data. The eigengene is defined as the first principal component of a given module and it represents a measure of miRNA expression profiles in the module. A module was chosen for further analysis if it presented a module−trait relationship > |0.5| and a *P*-value < 0.05. Potential biologically interesting (significant) modules were selected for downstream analysis. Furthermore, miRNAs in selected modules were used for functional enrichment analysis if eigengene−based connectivity (k.ME), a measure of how the miRNA is correlated to module eigengene, was > 0.6 (k.ME > 0.6)(Alexandre et al., 2015). A k.ME > 0.6 indicates higher connectivity and thus higher representation of modular functions.

3.3.8 Predicted Target mRNAs of miRNAs

In order to investigate the function of miRNAs in modules significantly correlated with traits, we first predicted their target mRNAs. The perl scripts from the TargetScan website (http://targetscan.org) were used to predict target mRNAs (targetscan_60.pl) and also to calculate their context scores (targetscan_61_context_scores.pl). TargetScan computes the context++ score for a specific site as the sum of the contribution of 14 features of the miRNA, miRNA site, or mRNA (including the mRNA surrounding sequence) (http://www.targetscan.org/vert_70/docs/context_score.html) to define sites on mRNAs most effectively targeted by miRNAs (Agarwal et al., 2015) Predicted target mRNAs with context ++ scores above 95th percentile were further used (Li et al., 2015; Do et al., 2017; Do et al., 2017). The predicted target mRNAs were then filtered against the mRNA expression data from the same animals (Ibeagha-Awemu et al., 2016). Only target mRNAs that were present in the mRNA expression data were retained for further analysis.

3.3.9 Co-Expression Analysis of miRNA–mRNA Expression

For miRNA–mRNA co-expression, the Pearson correlation coefficient between target mRNAs and miRNAs were calculated. A miRNA–mRNA pair was considered co-expressed if it had a negative and significant correlation value at FDR < 0.05. To further explore how miRNAs contributed to particular traits, we examined the correlation between miRNAs and their mRNA targets with the phenotypes in each significantly correlated module. Important interactions between miRNA and mRNAs were visualized using Cytoscape (Shannon et al., 2003).

3.3.10 Gene Ontologies, Pathways and Transcription Factors Enrichment

Functional enrichment of GO terms of target mRNAs was performed for each selected module using EnrichR (Chen et al., 2013; Kuleshov et al., 2016). EnrichR presents results according to hierarchy and relationship between terms which facilitates the interpretation of results. In this enrichment, the *P* -values for each term were adjusted using Benjamini–Hochberg (BH) correction(Chen et al., 2013). Gene ontology terms, KEGG pathways and TF were considered significantly enriched at adjusted $P < 0.05$ (FDR < 0.05).

3.4 Results

3.4.1 Phenotypic data

A summary of the data on blood metabolites, milk and component yields including fatty acid profiles for the control and treatment periods used for co-expression and network analyses is shown in Table 3.1.

Table 3.1 Means (± standard error) of phenotypic data used for co-expression network analyses*.

Traits	Names	Unit	Control			Linseed oil treatment			Safflower oil treatment		
			Mean ± se	min	max	Mean ± se	min	max	Mean ± se	min	max
PROT_Y	Protein yield	kg	1.2±0.06	0.99	1.52	1.24±0.06	0.92	1.53	1.17±0.06	0.89	1.47
FAT_Y	Fat yield	kg	1.31±0.08	1.12	1.74	1.07±0.11	0.61	1.98	0.97±0.07	0.67	1.35
Milk	Milk yield	kg	36.94±2.13	29.24	52.24	38.14±2.42	26.44	53.56	36.64±2.86	26.16	53.34
PRT	Protein percentage	%	3.3±0.1	2.9	3.65	3.31±0.11	2.81	4.17	3.27±0.1	2.74	3.85
LAC	Lactose percentage	%	4.7±0.05	4.47	4.88	4.74±0.05	4.43	4.94	4.65±0.04	4.42	4.84
FAT	Fat percentage	%	3.6±0.12	3.07	4.19	2.8±0.19	1.87	3.7	2.77±0.24	1.42	3.99
TAG	Triacylglyceride	nmol/L	0.05±0	0.02	0.07	0.08±0.01	0.04	0.11	0.08±0.01	0.05	0.12
NEFA	non-esterified fatty acids	nmol/L	103.86±23.55	48.66	312.99	156.48±13.29	92.75	248.53	154.47±14.72	101.13	276.18
C4:0	Butyric acid	mg/100g of fat	2.81±0.35	1.51	4.95	0.64±0.1	0.06	0.95	0.74±0.08	0.03	0.94
C6:0	Caproic acid	mg/100g of fat	2.39±0.18	1.31	3.58	0.97±0.15	0.28	1.51	1.24±0.16	0.52	2.26
C8:0	Caprylic acid	mg/100g of fat	1.18±0.07	1.05	1.66	0.72±0.06	0.5	0.94	0.76±0.05	0.51	1.04
C12:0	Lauric acid	mg/100g of fat	2.77±0.3	1.01	4.68	1.5±0.24	0.56	3.03	1.44±0.21	0.5	2.93
C14:0	Myristic acid	mg/100g of fat	11.43±0.27	10.22	12.7	7.95±0.41	5.47	9.28	6.96±0.51	5.01	9.86
C15:0	Pentadecylic acid	mg/100g of fat	1.44±0.07	1.13	1.86	0.98±0.07	0.68	1.4	0.99±0.05	0.8	1.4

C16:0	Palmitic acid	mg/100g of fat	26.79±0.28	25.46	28.55	21.17±1.2	16.64	28.7	21.94±0.98	17.22	26.7
C17:0	Margaric acid	mg/100g of fat	0.83±0.1	0.44	1.73	0.93±0.12	0.36	1.49	0.73±0.1	0.16	1.29
C18:0	Stearic acid	mg/100g of fat	11.13±0.54	8.02	13.87	8.58±0.49	5.02	10.41	7.57±0.37	5.5	9.02
C20:0	Arachidic acid	mg/100g of fat	0.22±0.03	0.1	0.34	0.21±0.02	0.12	0.29	0.21±0.02	0.1	0.35
C22:0	Behenic acid	mg/100g of fat	0.05±0	0.02	0.07	0.04±0	0.02	0.05	0.04±0	0.03	0.05
C23:0	Tricosanoic acid	mg/100g of fat	0.04±0	0.02	0.05	0.03±0	0.01	0.06	0.04±0	0.02	0.05
C24:0	Lignoceric acid	mg/100g of fat	0.04±0	0.03	0.05	0.03±0	0.01	0.04	0.03±0	0.01	0.05
C14:1total	Myristoleic acid	mg/100g of fat	11±-1.13	0.28	1.2	10±-1.6	0.25	1.02	11±-1.79	0.46	0.98
C16:1total	Palmitoleic acid	mg/100g of fat	1.31±0.06	1.1	1.71	1.65±0.23	0.42	3.08	1.82±0.18	1.21	2.84
C18:1n9c	Oleic acid	mg/100g of fat	19.06±1.35	10.7	24	25.91±1.12	20.08	33.2	22.57±0.7	19.4	26
C20:2	Eicosadienoic acid	mg/100g of fat	0.03±0	0.02	0.05	0.07±0.01	0.01	0.14	0.08±0.01	0.04	0.13
C22:5n3	Docosapentaenoic acid	mg/100g of fat	0.06±0.01	0.02	0.16	0.23±0.04	0.04	0.47	0.14±0.03	0.03	0.32
C22:6n3	Docosahexaenoic acid	mg/100g of fat	0.14±0	0.12	0.16	0.18±0.01	0.13	0.23	0.18±0.01	0.12	0.26
C18:3n3n	Alpha linolenic acid	mg/100g of fat	0.27±0.02	0.2	0.42	0.32±0.02	0.19	0.4	0.21±0.03	0.1	0.49
CLA:9,11	*cis*-9, *trans*-11 CLA	mg/100g of fat	0.3±0.02	0.16	0.41	0.33±0.02	0.22	0.39	0.31±0.04	0.15	0.54
CLA:10,12	*Trans*-10, *cis*-12 CLA	mg/100g of fat	0.02±0	0.01	0.03	0.04±0	0.03	0.07	0.04±0	0.02	0.06
MUFA	Sum of Monounsaturated fatty acids	mg/100g of fat	22.99±1.38	15.18	28.33	30.59±1.17	24.53	37.83	27.55±0.9	24.32	32.59

SFA	Sum of saturated fatty acids	mg/100g of fat	61.12±0.96	57.42	66.05	43.73±1.49	36.32	51.9	42.7±1.49	35.61	49.1
PUFA	Sum of Polyunsaturated fatty acids	mg/100g of fat	0.84±0.03	0.63	0.99	1.17±0.07	0.82	1.52	0.96±0.1	0.57	1.63

*Data on traits are the means of data collected on days -14 and -1 for the control period and days +7, +14, +21 and +28 for the treatment period. This table presents the l effects of treatments or control on traits in the data used in the current study. Detailed results on the effects of treatments on data collected at the different time points have been described previously (Ammah et al., 2018; Ibeagha-Awemu et al., 2016)

3.4.2 Identification of Consensus Modules and Module Trait Relationship

Using the WGCNA approach for miRNA read data described by Li et al. [12], we identified a total of three consensus modules (blue, brown, and turquoise) of co-expressed miRNAs during the control and TP (Figures 3.1 and 3.2). The modules were made up of 70 (blue), 34 (brown), and 86 (turquoise) miRNA members (Figures 3.1 and 3.2). The grey module grouped miRNAs with no coherent co-expression patterns; therefore, it was not further discussed. MiRNAs were further selected based on their intra modular connectivity or eigengene-based connectivity (k.ME). The k.ME is a measure of how a miRNA is correlated to module eigengene and miRNAs with high k.ME values (>0.6) are better representatives of module characteristics (Horvath and Dong, 2008). Therefore, miRNAs with k.ME > 0.6 were selected for downstream analyses. A total of 18, 12 and 19 miRNAs with k.ME > 0.6 in the blue, brown, and turquoise modules, respectively, were selected for downstream analyses (Table 3.2). Hub miRNAs or miRNAs with the most connections with other members of the module were bta-miR-30d, bta-miR-484 and bta-miR-16b for the blue, brown, and turquoise modules, respectively (Table 3.2).

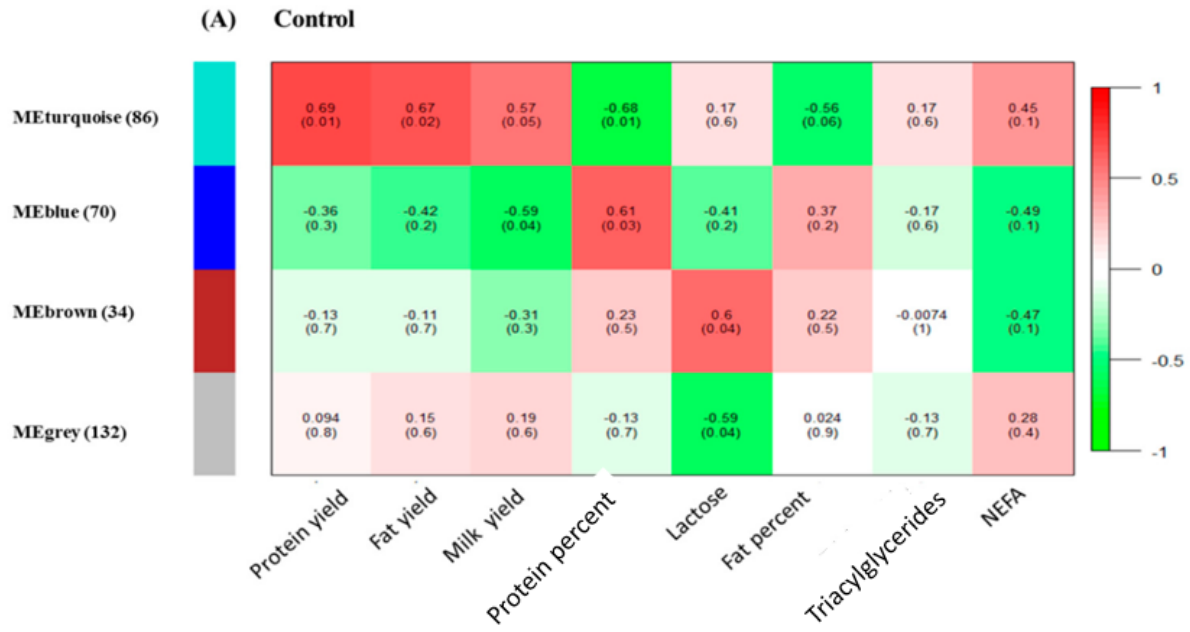

Figure 3.1 A

Consensus modules and module-trait relationship matrix: The weighted gene co-expression network analysis (WGCNA) was used to group miRNAs into consensus modules based on their expression patterns. Four consensus modules were identified and each consensus module eigengene was tested for correlation with blood and milk parameters during the CP. Correlation coefficients and corresponding *P* -values (in brackets) between turquoise, blue and brown modules in the *y*-axis, and blood and milk parameters in the *x*-axis. The module−trait relationship matrix is colored based on the intensity of the correlation: red is a strong positive correlation, while green is a strong negative correlation.

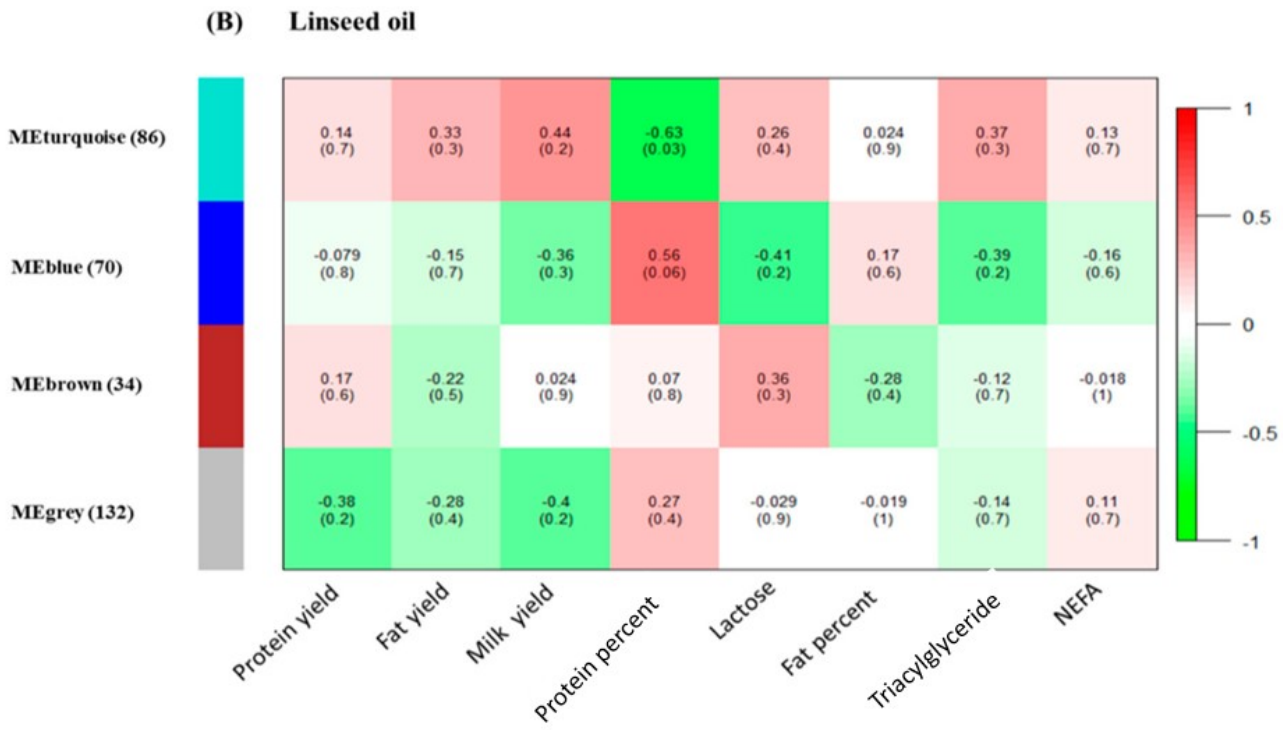

Figure 3.1 B

Consensus modules and module-trait relationship matrix: The weighted gene co-expression network analysis (WGCNA) was used to group miRNAs into consensus modules based on their expression patterns. Four consensus modules were identified and each consensus module eigengene was tested for correlation with blood and milk parameters during LSO treatment. Correlation coefficients and corresponding *P* -values (in brackets) between turquoise, blue and brown modules in the *y*-axis, and blood and milk parameters in the *x*-axis. The module−trait relationship matrix is colored based on the intensity of the correlation: red is a strong positive correlation, while green is a strong negative correlation.

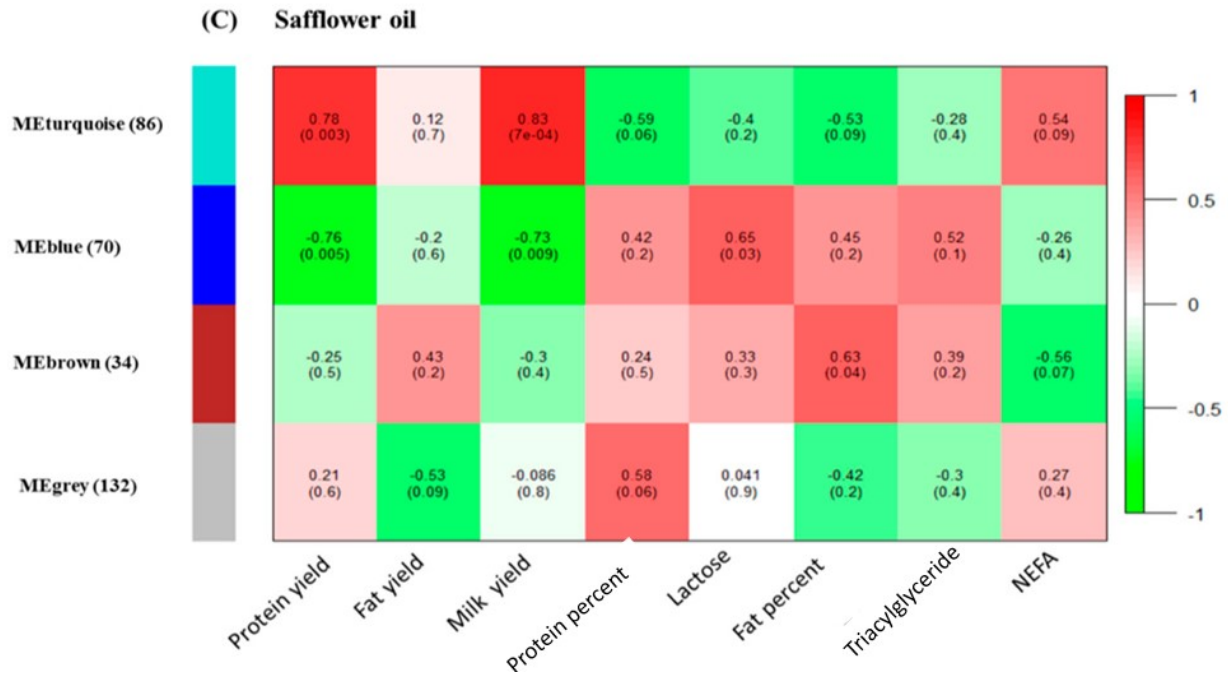

Figure 3.1 C

Consensus modules and module-trait relationship matrix: The weighted gene co-expression network analysis (WGCNA) was used to group miRNAs into consensus modules based on their expression patterns. Four consensus modules were identified and each consensus module eigengene was tested for correlation with blood and milk parameters during SFO treatment. Correlation coefficients and corresponding *P* -values (in brackets) between turquoise, blue and brown modules in the *y*-axis, and blood and milk parameters in the *x*-axis. The module−trait relationship matrix is colored based on the intensity of the correlation: red is a strong positive correlation, while green is a strong negative correlation.

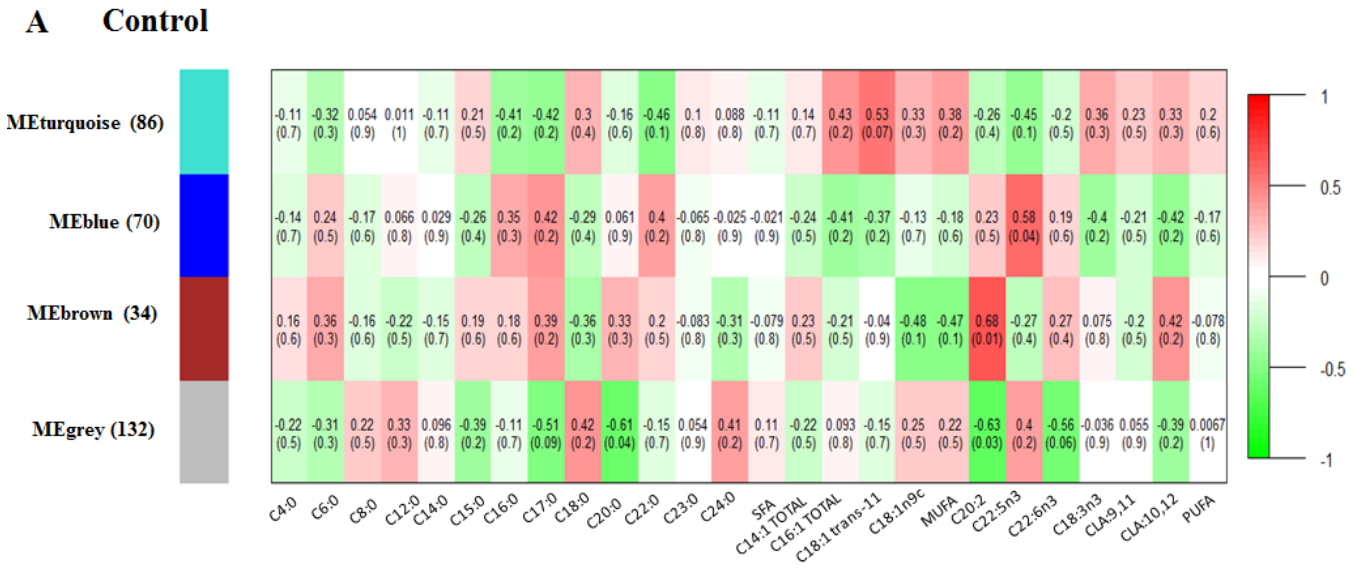

Figure 3.2 A

Consensus module−trait relationship matrix: The weighted gene co-expression network analysis (WGCNA) was used to group miRNAs into consensus modules based on their expression patterns. Four consensus modules were identified and each consensus module eigengene was tested for correlation with milk fatty acid traits during control period. Correlation coefficients and corresponding *P* -values (in brackets) between miRNA modules in the *y*-axis and milk fatty acids in the *x*-axis. The module−trait relationship matrix is colored based on the intensity of the correlation: red is a strong positive correlation, while green is a strong negative correlation.

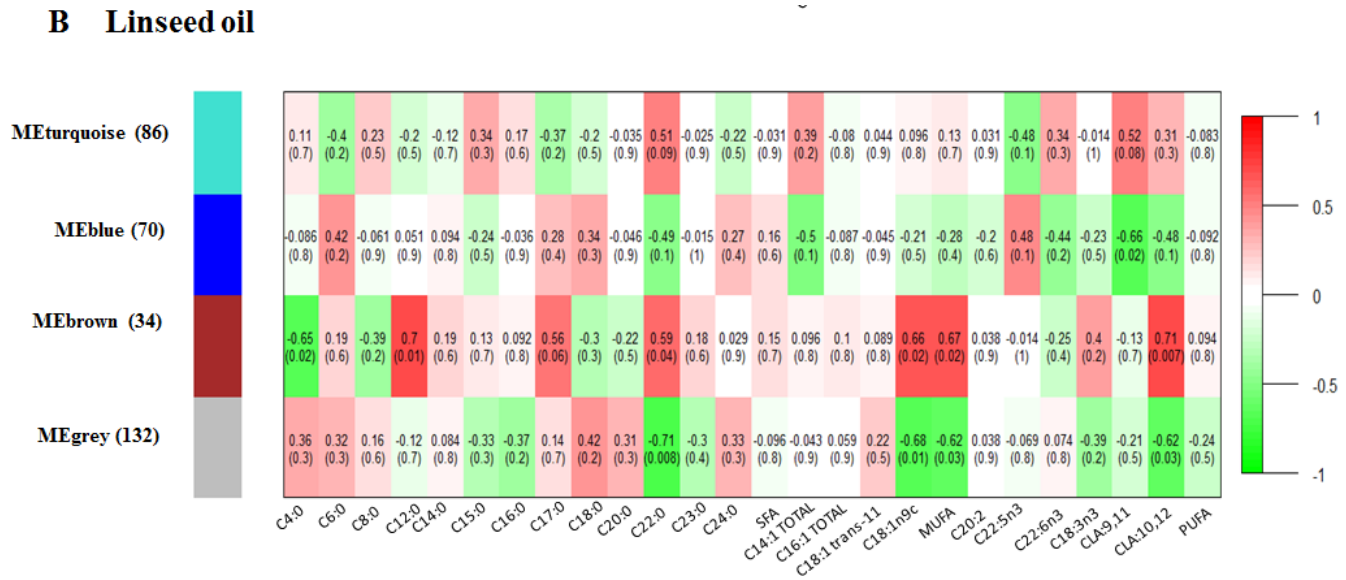

Figure 3.2 B

Consensus module−trait relationship matrix: The weighted gene co-expression network analysis (WGCNA) was used to group miRNAs into consensus modules based on their expression patterns. Four consensus modules were identified and each consensus module eigengene was tested for correlation with milk fatty acid traits during LSO treatment. Correlation coefficients and corresponding *P* -values (in brackets) between miRNA modules in the *y*-axis and milk fatty acids in the *x*-axis. The module−trait relationship matrix is colored based on the intensity of the correlation: red is a strong positive correlation, while green is a strong negative correlation.

C Safflower oil

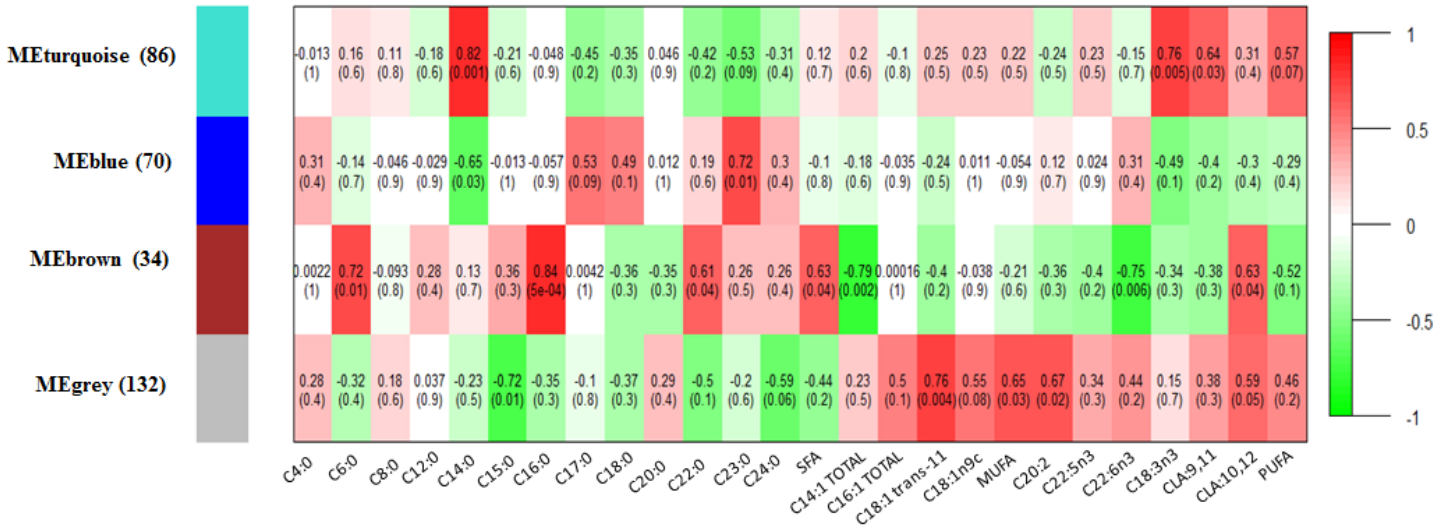

Figure 3.2 C

Consensus module−trait relationship matrix: The weighted gene co-expression network analysis (WGCNA) was used to group miRNAs into consensus modules based on their expression patterns. Four consensus modules were identified and each consensus module eigengene was tested for correlation with milk fatty acid traits during SFO treatment. Correlation coefficients and corresponding *P* -values (in brackets) between miRNA modules in the *y*-axis and milk fatty acids in the *x*-axis. The module−trait relationship matrix is colored based on the intensity of the correlation: red is a strong positive correlation, while green is a strong negative correlation.

Table 3.2 Consensus modules (blue, brown and turquoise) and their miRNA members.

Module	miRNA	[1]k.ME_All	*P*-value k.ME_All	[2]k.ME_Control	*P*-value k.ME_Control	[3]k.ME_Linseed	*P*-value k.ME_Linseed	[4]k.ME_Safflower	*P*-value k.ME_Safflower
Blue	bta-miR-30d	0.93	1.58E-22	0.96	5.06E-07	0.95	1.24E-06	0.93	1.69E-05
Blue	bta-miR-96	0.89	1.62E-15	0.89	6.36E-05	0.90	2.62E-05	0.90	6.64E-05
Blue	bta-miR-191	0.87	4.00E-20	0.93	5.88E-06	0.97	9.66E-08	0.87	2.89E-04
Blue	bta-miR-151-5p	0.83	1.04E-14	0.90	2.68E-05	0.92	1.50E-05	0.83	7.43E-04
Blue	bta-miR-409a	0.80	6.90E-12	0.85	2.47E-04	0.80	9.23E-04	0.89	1.10E-04
Blue	bta-miR-183	0.77	4.79E-13	0.90	4.18E-05	0.77	1.79E-03	0.91	5.11E-05
Blue	bta-miR-99a-5p	0.77	1.49E-14	0.91	1.82E-05	0.77	1.79E-03	0.94	1.20E-05
Blue	bta-let-7b	0.76	1.66E-09	0.76	2.24E-03	0.80	8.20E-04	0.84	6.75E-04
Blue	bta-miR-2285k	0.75	2.89E-09	0.75	2.29E-03	0.75	2.71E-03	0.86	2.96E-04
Blue	bta-miR-652	0.73	1.21E-11	0.90	2.88E-05	0.86	1.59E-04	0.73	5.76E-03
Blue	bta-let-7a-5p	0.70	9.32E-09	0.81	7.38E-04	0.81	6.60E-04	0.70	8.10E-03
Blue	bta-miR-6522	0.68	3.17E-08	0.74	2.95E-03	0.84	2.87E-04	0.68	1.12E-02
Blue	bta-miR-100	0.68	2.69E-08	0.77	1.57E-03	0.68	7.88E-03	0.83	7.85E-04
Blue	bta-miR-374a	0.66	8.19E-08	0.77	1.72E-03	0.66	9.28E-03	0.80	1.46E-03
Blue	bta-miR-2284b	0.66	2.57E-07	0.66	9.38E-03	0.76	2.06E-03	0.76	3.07E-03
Blue	bta-miR-532	0.65	1.39E-07	0.83	4.06E-04	0.65	1.05E-02	0.71	7.23E-03
Blue	bta-miR-99b	0.64	1.01E-10	0.89	4.73E-05	0.64	1.28E-02	0.88	1.92E-04
Blue	bta-miR-23b-3p	0.62	2.65E-07	0.77	1.59E-03	0.62	1.66E-02	0.78	2.15E-03
Brown	bta-miR-484	0.78	1.28E-11	0.86	1.77E-04	0.78	1.27E-03	0.88	1.68E-04
Brown	bta-let-7d	0.76	1.24E-13	0.89	6.15E-05	0.93	6.37E-06	0.76	3.08E-03
Brown	bta-miR-429	0.74	8.47E-12	0.74	3.16E-03	0.87	1.13E-04	0.90	7.33E-05
Brown	bta-miR-885	0.73	2.27E-11	0.94	4.02E-06	0.77	1.57E-03	0.73	5.48E-03
Brown	bta-miR-26b	0.72	5.57E-09	0.74	2.98E-03	0.87	1.32E-04	0.72	6.31E-03

Brown	bta-miR-30c	0.71	4.04E-13	0.71	4.60E-03	0.93	5.77E-06	0.89	1.03E-04
Brown	bta-let-7g	0.70	2.80E-08	0.82	5.04E-04	0.70	5.68E-03	0.76	3.39E-03
Brown	bta-miR-29b	0.64	7.43E-06	0.68	7.75E-03	0.64	1.26E-02	0.68	1.03E-02
Brown	bta-miR-328	0.63	1.02E-06	0.63	1.39E-02	0.81	6.48E-04	0.64	1.67E-02
Brown	bta-miR-32	0.63	2.25E-07	0.63	1.43E-02	0.78	1.51E-03	0.78	2.35E-03
Brown	bta-miR-107	0.61	4.32E-07	0.61	1.78E-02	0.77	1.79E-03	0.77	2.66E-03
Brown	bta-let-7a-3p	0.60	2.14E-08	0.83	4.16E-04	0.82	5.65E-04	0.60	2.51E-02
Turquoise	bta-miR-16b	0.85	4.24E-12	0.86	1.93E-04	0.86	1.85E-04	0.85	4.94E-04
Turquoise	bta-miR-130a	0.84	1.36E-15	0.95	1.78E-06	0.88	6.75E-05	0.84	6.51E-04
Turquoise	bta-miR-142-5p	0.84	2.35E-13	0.88	7.90E-05	0.89	4.54E-05	0.84	6.66E-04
Turquoise	bta-miR-218	0.81	2.47E-14	0.85	2.40E-04	0.81	7.09E-04	0.95	3.45E-06
Turquoise	bta-miR-142-3p	0.80	9.04E-13	0.89	5.46E-05	0.88	7.17E-05	0.80	1.40E-03
Turquoise	bta-miR-195	0.77	1.20E-12	0.77	1.55E-03	0.80	8.03E-04	0.95	5.53E-06
Turquoise	bta-miR-497	0.75	5.71E-13	0.75	2.29E-03	0.85	2.52E-04	0.94	7.26E-06
Turquoise	bta-miR-16a	0.74	2.61E-11	0.82	5.21E-04	0.74	2.91E-03	0.92	3.83E-05
Turquoise	bta-miR-19b	0.70	1.90E-08	0.81	6.35E-04	0.70	5.93E-03	0.79	1.95E-03
Turquoise	bta-miR-3613	0.68	4.50E-07	0.76	2.12E-03	0.68	7.00E-03	0.72	6.31E-03
Turquoise	bta-miR-455-3p	0.67	1.02E-06	0.67	8.79E-03	0.70	5.94E-03	0.76	3.60E-03
Turquoise	bta-miR-15a	0.66	1.15E-07	0.85	2.03E-04	0.67	8.90E-03	0.66	1.30E-02
Turquoise	bta-miR-424-5p	0.65	1.45E-08	0.65	1.07E-02	0.89	5.66E-05	0.71	6.99E-03
Turquoise	bta-miR-106b	0.64	4.81E-12	0.89	5.64E-05	0.64	1.18E-02	0.93	2.05E-05
Turquoise	bta-miR-155	0.64	2.68E-07	0.83	4.77E-04	0.69	6.30E-03	0.64	1.68E-02
Turquoise	bta-miR-455-5p	0.63	7.14E-07	0.67	8.50E-03	0.63	1.35E-02	0.81	1.14E-03
Turquoise	bta-miR-93	0.63	5.83E-10	0.91	1.70E-05	0.63	1.37E-02	0.80	1.54E-03
Turquoise	bta-miR-199a-5p	0.61	5.53E-06	0.71	4.58E-03	0.68	7.05E-03	0.61	2.27E-02
Turquoise	bta-miR-99a-3p	0.60	7.73E-07	0.66	9.23E-03	0.60	1.94E-02	0.83	7.22E-04

[1]Eigengene-based connectivity (k.ME), a correlation coefficient of miRNA expression and the module eigengene value in all samples. K.ME is a measure of how the miRNA is correlated to module eigengene. [2]Correlation coefficient of miRNA expression and the module eigengene value in control samples. [3]Correlation coefficient of miRNA expression and the module eigengene value in Linseed oil treatment. [4]Correlation coefficient of miRNA expression and the module eigengene value in Safflower oil treatment.

In LSO treatment, the turquoise module correlated significantly with protein percent ($P = 0.03$) while protein yield ($P = 0.003$) and milk yield ($P = 7 \times 10^{-4}$) correlated with the turquoise module, protein and milk yields and lactose percent ($P < 0.05$) correlated with the blue module and fat percent ($P = 0.04$) correlated with the brown module in SFO treatment (Figure 3.1). Several fatty acids correlated ($P < 0.05$) with the blue (CLA:9,11) and brown (C4:0, C12:0, C22:0, C18:1n9c and CLA:10,12) modules in LSO treatment and with the turquoise (C14:0, C18:3n3 and CLA:9,11), blue (C14:0 and C23:0) and brown (C6:0, C16:0, C22:0, C22:6n3 and CLA:10,12) modules in SFO treatment (Figure 3.2). Several measured parameters also correlated with the identified modules in the control samples (Figure 3.1 and Figure 3.2).

3.4.3 miRNA Target Gene Prediction and Enrichment Analysis

Using TargetScan, we identified 3199, 3727, and 4045 target mRNAs for 18, 12, and 19 miRNAs in the blue, brown, and turquoise modules, respectively (Table S1a,c,e). Amongst them, 1311, 1533, and 1697 mRNAs from mRNA data of the same samples (Ibeagha-Awemu et al., 2016) had significant negative correlations (FDR < 0.05) with 18, 12 and 19 miRNAs in the blue, brown, and turquoise modules, respectively (Table S1b,d,e). These mRNAs (filtered target mRNAs) were used as input for enrichment analyses for GO, KEGG pathways and TF. A total of 6, 16, and 84 GO terms were enriched for filtered target mRNAs of the blue, brown, and turquoise modules, respectively (Table 3.3). The GO term, cell cycle arrest (GO: 0007050) was common to the three modules (Figure 3.3, Table 3.3). Vesicle docking (GO: 0048278), GDP binding (*GO: 0019003)* and GTP binding (*GO: 0005525*) were the most significantly enriched GO terms for the blue, brown and turquoise modules, respectively (Table 3.3).

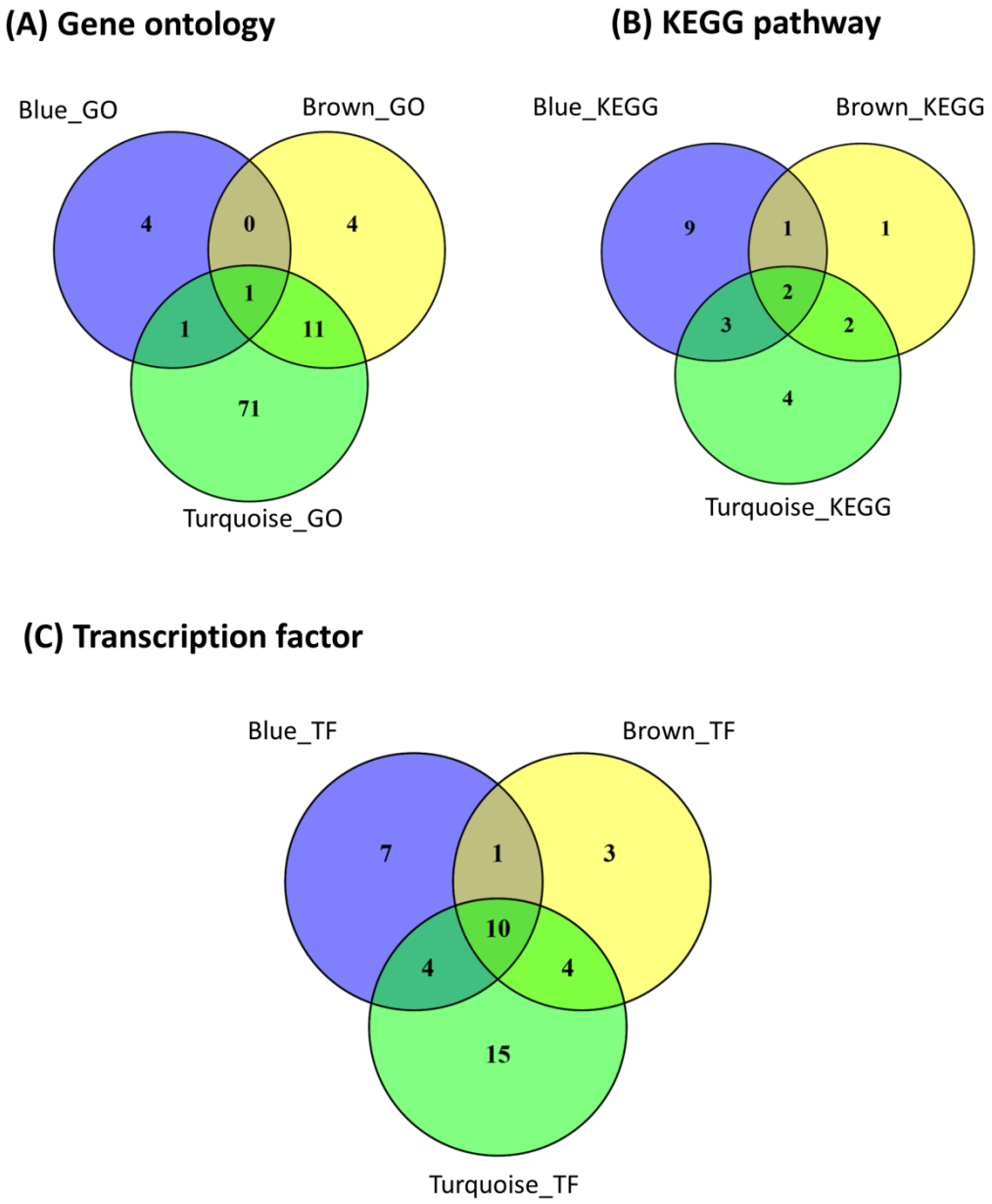

Figure 3.3 Venn diagrams showing number of enriched unique and shared (a) gene ontology terms, (b) KEGG pathways and (c) transcription factors for predicted target genes of miRNA in the blue, brown and turquoise modules. GO: Gene ontology, KEGG; KEGG pathway, TF: Transcription factor.

Table 3.3 Enriched gene ontology (GO) terms for the blue, brown, and turquoise modules.

Modules	Term	GO ID	*P*-value	Adjusted *P*-value*
Blue	Vesicle docking	GO:0048278	8.02E-06	1.53E-02
Blue	Negative regulation of transcription from RNA Polymerase II promoter	GO:0000122	2.49E-05	2.38E-02
Blue	Proteasome-mediated ubiquitin-dependent protein catabolic process	GO:0043161	5.12E-05	2.44E-02
Blue	Protein dephosphorylation	GO:0006470	4.44E-05	2.44E-02
Blue	Cell cycle arrest	GO:0007050	1.02E-04	3.69E-02
Blue	RNA splicing	GO:0008380	1.16E-04	3.69E-02
Brown	GDP binding	GO:0019003	1.16E-09	6.63E-07
Brown	GTP binding	GO:0005525	2.35E-08	6.71E-06
Brown	GTPase activity	GO:0003924	9.34E-08	1.78E-05
Brown	RNA binding	GO:0003723	4.05E-06	5.78E-04
Brown	Transforming growth factor beta receptor signaling pathway	GO:0007179	5.82E-07	1.19E-03
Brown	Protein serine/threonine kinase activity	GO:0004674	1.72E-05	1.40E-03
Brown	Transforming growth factor beta binding	GO:0050431	1.52E-05	1.40E-03
Brown	Transforming growth factor beta-activated receptor activity	GO:0005024	1.48E-05	1.40E-03
Brown	Peptidyl-prolyl *cis*-trans isomerase activity	GO:0003755	6.38E-05	4.56E-03
Brown	Ubiquitin protein ligase activity	GO:0061630	8.32E-05	5.28E-03
Brown	Type I transforming growth factor beta receptor binding	GO:0034713	1.21E-04	6.91E-03
Brown	mRNA splicing, via spliceosome	GO:0000398	7.53E-06	7.70E-03
Brown	Activin binding	GO:0048185	4.78E-04	2.48E-02
Brown	Protein ubiquitination	GO:0016567	4.28E-05	2.92E-02
Brown	Ubiquitin-protein transferase activity	GO:0004842	7.29E-04	3.47E-02
Brown	Cell cycle arrest	GO:0007050	9.44E-05	4.83E-02
Turquoise	GTP binding	GO:0005525	6.32E-10	4.13E-07
Turquoise	Macroautophagy	GO:0016236	2.44E-10	5.31E-07
Turquoise	Proteasome-mediated ubiquitin-dependent protein catabolic process	GO:0043161	2.35E-09	2.56E-06
Turquoise	Membrane organization	GO:0061024	4.22E-09	3.06E-06
Turquoise	RNA binding	GO:0003723	4.15E-08	9.27E-06
Turquoise	GDP binding	GO:0019003	4.26E-08	9.27E-06
Turquoise	Transcription coactivator activity	GO:0003713	2.73E-07	3.82E-05

Turquoise	GTPase activity	GO:0003924	2.93E-07	3.82E-05
Turquoise	Ubiquitin protein ligase activity	GO:0061630	9.30E-07	1.01E-04
Turquoise	Ubiquitin protein ligase binding	GO:0031625	1.10E-06	1.03E-04
Turquoise	Protein serine/threonine kinase activity	GO:0004674	1.99E-06	1.62E-04
Turquoise	Protein K48-linked ubiquitination	GO:0070936	5.71E-07	3.10E-04
Turquoise	Protein ubiquitination involved in ubiquitin-dependent protein catabolic process	GO:0042787	9.25E-07	4.02E-04
Turquoise	Regulation of transcription from RNA polymerase II promoter	GO:0006357	1.44E-06	5.18E-04
Turquoise	Protein deubiquitination	GO:0016579	1.91E-06	5.18E-04
Turquoise	Nucleotide-excision repair, preincision complex assembly	GO:0006294	1.77E-06	5.18E-04
Turquoise	Cadherin binding	GO:0045296	1.10E-05	7.99E-04
Turquoise	Protein ubiquitination	GO:0016567	5.19E-06	1.25E-03
Turquoise	Protein homodimerization activity	GO:0042803	2.27E-05	1.48E-03
Turquoise	Protein polyubiquitination	GO:0000209	7.21E-06	1.57E-03
Turquoise	Ubiquitin-protein transferase activity	GO:0004842	3.22E-05	1.91E-03
Turquoise	Golgi organization	GO:0007030	9.86E-06	1.95E-03
Turquoise	Transforming growth factor beta receptor signaling pathway	GO:0007179	1.13E-05	2.04E-03
Turquoise	G2/M transition of mitotic cell cycle	GO:0000086	1.74E-05	2.90E-03
Turquoise	Negative regulation of apoptotic process	GO:0043066	2.37E-05	3.69E-03
Turquoise	Positive regulation of apoptotic process	GO:0043065	2.59E-05	3.75E-03
Turquoise	GABA receptor binding	GO:0050811	7.94E-05	4.32E-03
Turquoise	Global genome nucleotide-excision repair	GO:0070911	3.83E-05	5.12E-03
Turquoise	Stress-activated MAPK cascade	GO:0051403	4.00E-05	5.12E-03
Turquoise	Positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	GO:0051437	6.07E-05	6.95E-03
Turquoise	Protein K11-linked ubiquitination	GO:0070979	5.94E-05	6.95E-03
Turquoise	Protein phosphorylation	GO:0006468	6.40E-05	6.96E-03
Turquoise	Anaphase-promoting complex-dependent catabolic process	GO:0031145	1.03E-04	1.07E-02
Turquoise	Post-translational protein modification	GO:0043687	1.14E-04	1.11E-02
Turquoise	Virion assembly	GO:0019068	1.18E-04	1.11E-02
Turquoise	Transforming growth factor beta binding	GO:0050431	2.84E-04	1.32E-02

Turquoise	Cadherin binding involved in cell-cell adhesion	GO:0098641	2.84E-04	1.32E-02
Turquoise	Ligand-dependent nuclear receptor transcription coactivator activity	GO:0030374	3.41E-04	1.48E-02
Turquoise	ER to Golgi vesicle-mediated transport	GO:0006888	1.83E-04	1.66E-02
Turquoise	Protein kinase activity	GO:0004672	4.47E-04	1.69E-02
Turquoise	R-SMAD binding	GO:0070412	4.30E-04	1.69E-02
Turquoise	Ubiquitin conjugating enzyme binding	GO:0031624	4.67E-04	1.69E-02
Turquoise	Ubiquitin-dependent protein catabolic process	GO:0006511	2.03E-04	1.70E-02
Turquoise	COPII vesicle coating	GO:0048208	1.96E-04	1.70E-02
Turquoise	Thiol-dependent ubiquitinyl hydrolase activity	GO:0036459	5.21E-04	1.79E-02
Turquoise	Cellular response to DNA damage stimulus	GO:0006974	2.41E-04	1.87E-02
Turquoise	Endocytosis	GO:0006897	2.37E-04	1.87E-02
Turquoise	Negative regulation of sequence-specific DNA binding transcription factor activity	GO:0043433	2.51E-04	1.88E-02
Turquoise	Regulation of signal transduction by p53 class mediator	GO:1901796	2.80E-04	1.94E-02
Turquoise	Wnt signaling pathway, planar cell polarity pathway	GO:0060071	2.72E-04	1.94E-02
Turquoise	Nucleotide-excision repair, DNA duplex unwinding	GO:0000717	2.86E-04	1.94E-02
Turquoise	Transcription cofactor activity	GO:0003712	6.37E-04	2.08E-02
Turquoise	Polynucleotide adenylyltransferase activity	GO:0004652	6.74E-04	2.09E-02
Turquoise	Positive regulation of transcription from RNA polymerase II promoter	GO:0045944	3.39E-04	2.24E-02
Turquoise	Activin binding	GO:0048185	7.66E-04	2.27E-02
Turquoise	Androgen receptor signaling pathway	GO:0030521	3.64E-04	2.33E-02
Turquoise	BMP signaling pathway	GO:0030509	3.98E-04	2.47E-02
Turquoise	Guanyl-nucleotide exchange factor activity	GO:0005085	9.54E-04	2.71E-02
Turquoise	Double-stranded DNA binding	GO:0003690	1.10E-03	2.98E-02
Turquoise	Transcription from RNA polymerase II promoter	GO:0006366	5.41E-04	3.27E-02
Turquoise	Retrograde transport, endosome to plasma membrane	GO:1990126	6.11E-04	3.59E-02
Turquoise	Error-free translesion synthesis	GO:0070987	6.32E-04	3.62E-02
Turquoise	Endosomal transport	GO:0016197	6.73E-04	3.66E-02
Turquoise	GTP metabolic process	GO:0046039	6.74E-04	3.66E-02
Turquoise	NIK/NF-kappaB signaling	GO:0038061	7.95E-04	3.84E-02

Turquoise	Negative regulation of transforming growth factor beta receptor signaling pathway	GO:0030512	7.75E-04	3.84E-02
Turquoise	Negative regulation of cell death	GO:0060548	7.78E-04	3.84E-02
Turquoise	Nucleotide-excision repair, DNA incision, 5'-to lesion	GO:0006296	7.78E-04	3.84E-02
Turquoise	Negative regulation of actin filament polymerization	GO:0030837	7.66E-04	3.84E-02
Turquoise	Single-stranded DNA binding	GO:0003697	1.61E-03	4.19E-02
Turquoise	G1/S transition of mitotic cell cycle	GO:0000082	9.12E-04	4.22E-02
Turquoise	Negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	GO:0051436	9.00E-04	4.22E-02
Turquoise	Cell cycle arrest	GO:0007050	9.92E-04	4.40E-02
Turquoise	Nucleotide-excision repair, DNA incision	GO:0033683	9.77E-04	4.40E-02
Turquoise	Positive regulation of I-kappaB kinase/NF-kappaB signaling	GO:0043123	1.15E-03	4.86E-02
Turquoise	Regulation of small GTPase mediated signal transduction	GO:0051056	1.22E-03	4.86E-02
Turquoise	Regulation of transcription from RNA polymerase II promoter in response to hypoxia	GO:0061418	1.21E-03	4.86E-02
Turquoise	Wnt signaling pathway	GO:0016055	1.28E-03	4.86E-02
Turquoise	Autophagy	GO:0006914	1.31E-03	4.86E-02
Turquoise	Negative regulation of type I interferon production	GO:0032480	1.32E-03	4.86E-02
Turquoise	Nucleotide-excision repair	GO:0006289	1.32E-03	4.86E-02
Turquoise	Nucleotide-excision repair, preincision complex stabilization	GO:0006293	1.25E-03	4.86E-02
Turquoise	Nucleotide-excision repair, DNA incision, 3'-to lesion	GO:0006295	1.25E-03	4.86E-02
Turquoise	Alternative mRNA splicing, via spliceosome	GO:0000380	1.31E-03	4.86E-02

*Benjamini and Hochberg adjusted *P* -value

A total of 15, 6, and 11 KEGG pathways were enriched for the blue, brown, and turquoise modules, respectively (Table 3.4). Two KEGG pathways (p53 signaling and transforming growth factor (TGF) β signaling pathways) were common to the three modules (Table 3.4, Figure 3.3). Also, five (p53 signaling, cell cycle, Forkhead box O (FoxO) signaling, protein processing in endoplasmic reticulum and TGF-β signaling pathways) and four (TGF-β signaling, endocytosis, p53 signaling and ubiquitin mediated proteolysis pathways) pathways were common to the blue and turquoise, and brown and turquoise modules, respectively (Figure 3.3, Table 3.4). The most significantly enriched pathways in the blue, brown, and turquoise modules were p53 signaling pathway ($P = 9.93 \times 10^{-4}$), mitogen-activated protein kinase (MAPK) signaling pathway ($P = 3.40 \times 10^{-2}$) and ubiquitin mediated proteolysis pathway ($P = 5.81 \times 10^{-7}$), respectively.

Table 3.4 Enriched KEGG pathways for identified blue, brown and turquoise modules.

Module	Pathway	Pathway ID	*P* -value	Adjusted *P* -value*
Blue	p53 signaling pathway	hsa04115	7.33E-06	9.93E-04
Blue	Cell cycle	hsa04110	5.05E-06	9.93E-04
Blue	Proteoglycans in cancer	hsa05205	6.12E-05	5.53E-03
Blue	HTLV-I infection	hsa05166	1.77E-04	1.20E-02
Blue	Epstein-Barr virus infection	hsa05169	3.27E-04	1.78E-02
Blue	ErbB signaling pathway	hsa04012	4.90E-04	2.21E-02
Blue	MAPK signaling pathway	hsa04010	6.61E-04	2.38E-02
Blue	Chronic myeloid leukemia	hsa05220	8.32E-04	2.38E-02
Blue	Huntington's disease	hsa05016	8.52E-04	2.38E-02
Blue	Wnt signaling pathway	hsa04310	9.63E-04	2.38E-02
Blue	TGF-beta signaling pathway	hsa04350	1.05E-03	2.38E-02
Blue	Hippo signaling pathway	hsa04390	1.02E-03	2.38E-02
Blue	Pathways in cancer	hsa05200	1.49E-03	3.07E-02
Blue	Protein processing in endoplasmic reticulum	hsa04141	1.59E-03	3.07E-02
Blue	FoxO signaling pathway	hsa04068	2.64E-03	4.78E-02
Brown	MAPK signaling pathway	hsa04010	2.73E-04	3.40E-02

Brown	TGF-beta signaling pathway	hsa04350	1.83E-04	3.40E-02
Brown	Endocytosis	hsa04144	7.46E-04	3.40E-02
Brown	RNA degradation	hsa03018	6.58E-04	3.40E-02
Brown	p53 signaling pathway	hsa04115	6.43E-04	3.40E-02
Brown	Ubiquitin mediated proteolysis	hsa04120	7.30E-04	3.40E-02
Turquoise	Ubiquitin mediated proteolysis	hsa04120	2.15E-09	5.81E-07
Turquoise	Endocytosis	hsa04144	7.66E-08	1.03E-05
Turquoise	Protein processing in endoplasmic reticulum	hsa04141	8.37E-05	7.53E-03
Turquoise	p53 signaling pathway	hsa04115	1.69E-04	1.14E-02
Turquoise	Renal cell carcinoma	hsa05211	3.39E-04	1.83E-02
Turquoise	Focal adhesion	hsa04510	4.32E-04	1.94E-02
Turquoise	TGF-beta signaling pathway	hsa04350	6.00E-04	2.31E-02
Turquoise	Regulation of autophagy	hsa04140	1.22E-03	4.10E-02
Turquoise	FoxO signaling pathway	hsa04068	1.83E-03	4.48E-02
Turquoise	Nucleotide excision repair	hsa03420	1.58E-03	4.48E-02
Turquoise	Cell cycle	hsa04110	1.70E-03	4.48E-02

*Benjamini-Hochberg corrected *P* -values.

A total of 22, 18 and 33 TF were enriched for the blue, brown, and turquoise modules ($P < 0.05$), respectively (figure 3.3 and Table 3.5). Ten TF (*SMAD4, SP1, EGR1, NRF1*, *STAT3*, *E2F1*, *TP53*, *MEF2A*, *ATF4*, and *HIF1A*) were common to the three modules. Also, four (*NFYA*, *LEF1*, *PCBP1* and *ATF2*) TF were common to the blue and turquoise modules and another four (*PLAU*, *THRB*, *E2F6* and *TEAD4*) were common to the brown and turquoise modules. *SMAD4* was the most significantly enriched TF for the blue ($P = 1.28 \times 10^{-7}$) and turquoise ($P = 3.85 \times 10^{-11}$) modules; meanwhile, *SP1* was the most significantly enriched ($P = 2.41 \times 10^{-8}$) TF for the brown module.

Table 3.5 Enriched transcription factors for the blue, brown, and turquoise modules.

Module	Transcription factor	*P*-value	*FDR
Blue	SMAD4	4.27E-10	1.28E-07
Blue	SP1	1.95E-09	2.93E-07
Blue	EGR1	3.50E-08	3.50E-06
Blue	ZBTB16	2.99E-05	2.24E-03
Blue	STAT3	1.52E-04	7.59E-03
Blue	CBFB	2.60E-04	1.11E-02
Blue	TP53	3.32E-04	1.24E-02
Blue	FOXJ1	5.05E-04	1.52E-02
Blue	NFYA	4.81E-04	1.52E-02
Blue	NRF1	5.86E-04	1.57E-02
Blue	LEF1	6.28E-04	1.57E-02
Blue	SRF	1.00E-03	2.31E-02
Blue	PPARG	1.33E-03	2.85E-02
Blue	E2F1	2.32E-03	4.63E-02
Brown	SP1	8.01E-11	2.41E-08
Brown	EGR1	1.30E-06	1.30E-04
Brown	SMAD4	1.12E-06	1.30E-04
Brown	TP53	1.90E-05	1.43E-03
Brown	E2F1	2.75E-05	1.65E-03
Brown	PLAU	5.86E-05	2.94E-03
Brown	NRF1	1.50E-04	6.46E-03
Brown	THRB	1.94E-04	7.29E-03
Brown	NRF1	2.20E-04	7.35E-03
Brown	ATF4	4.00E-04	1.21E-02
Brown	HIF1A	6.13E-04	1.68E-02
Brown	E2F6	9.46E-04	2.37E-02
Brown	CREM	1.63E-03	3.76E-02
Brown	STAT3	2.02E-03	4.34E-02
Turquoise	SMAD4	1.22E-13	3.85E-11
Turquoise	SP1	3.05E-11	4.82E-09
Turquoise	E2F1	1.40E-05	8.86E-04
Turquoise	SP4	2.76E-05	1.46E-03
Turquoise	THRB	6.70E-05	3.02E-03
Turquoise	NRF1	1.77E-04	7.01E-03
Turquoise	LEF1	2.24E-04	7.86E-03
Turquoise	MEF2A	5.02E-04	1.59E-02
Turquoise	CEBPD	5.97E-04	1.66E-02
Turquoise	GATA1	6.32E-04	1.66E-02

Turquoise	ATF4	7.62E-04	1.76E-02
Turquoise	ATF2	8.38E-04	1.76E-02
Turquoise	NFYA	8.29E-04	1.76E-02
Turquoise	IRF8	1.17E-03	2.32E-02
Turquoise	NFAT2	1.39E-03	2.45E-02
Turquoise	STAT3	1.39E-03	2.45E-02
Turquoise	ELK4	2.03E-03	3.21E-02
Turquoise	TCFAP2A	1.97E-03	3.21E-02
Turquoise	PITX1	2.37E-03	3.56E-02
Turquoise	E2F6	2.76E-03	3.97E-02
Turquoise	SPI1	2.92E-03	4.02E-02
Turquoise	GTF2I	3.26E-03	4.02E-02
Turquoise	MAX	3.24E-03	4.02E-02
Turquoise	TEAD4	3.31E-03	4.02E-02
Turquoise	HNF1A	3.87E-03	4.40E-02
Turquoise	HINFP	4.13E-03	4.50E-02

*Benjamini-Hochberg corrected *P* -values.

3.4.4 Integration of miRNA–mRNA and Trait Relationship

A total of 132 of 1311 filtered target mRNAs, 19 of 1533 filtered target mRNAs and 2 of 1697 filtered target mRNAs were negatively and significantly correlated with miRNAs in the blue, brown, and turquoise modules, respectively (Table S1g). The most significantly correlated miRNA–mRNA pairs were bta-miR-183/*RHBDD2* ($P = 0.003$), bta-miR-484/*ADM2* ($P = 0.001$) and bta-miR-130a/*SBSPON* ($P = 0.004$) in the blue, brown, and turquoise modules, respectively. Moreover, 68, 6 and 2 miRNA–mRNA pairs were found to be significantly correlated with at least one phenotype at FDR < 0.05 (Table 3.6, Figure 3.4). Milk yield, protein yield, and protein percentage were correlated (FDR < 0.05) with 28, 31 and 5 miRNA–mRNA pairs, respectively (Table 3.6, Figure 3.4).

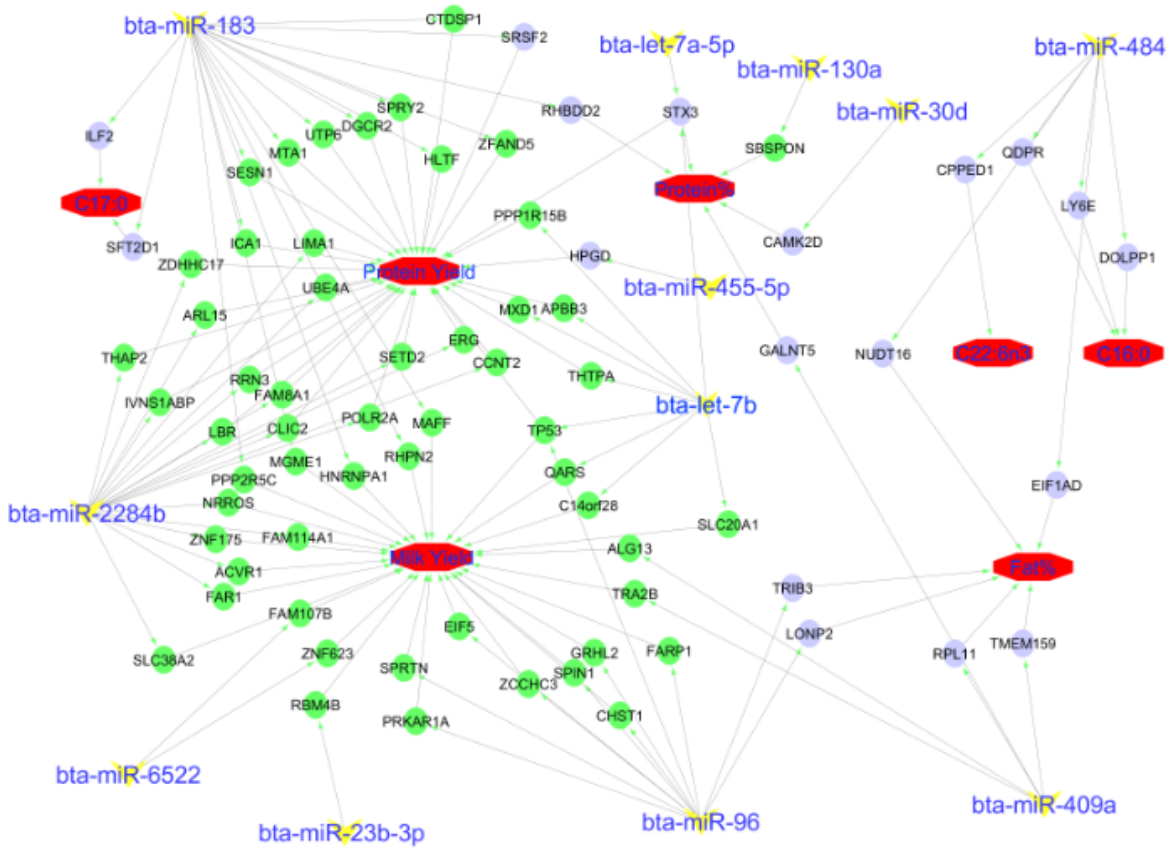

Figure 3.4 miRNA–mRNA-trait relationships. The mRNAs (green circles (negative correlation) or blue circles [positive correlation]) were significantly correlated with traits (octagonal red shapes). MiRNA ('Y' yellow shapes) were significantly correlated with traits (positively or negatively) and negatively correlated with corresponding mRNAs.

Table 3.6 Correlation of significant miRNA-mRNA pairs with phenotypes

Module	miRNA	Gene Symbol	[1]Context ++ score percentile	[2]Cor.mir.gene	[3]FDR.cor.mir.gene	Trait	[4]Cor.trait.mir	[5]FDR .cor.trait.mir	[6]Cor.gene.trait	[7]FDR.cor.gene.trait
Blue	bta-let-7a-5p	STX3	97	-0.428	0.022	Protein percentage	0.484	0.010	-0.438	0.023
Blue	bta-let-7b	*APBB3*	98	-0.394	0.037	Protein yield	-0.400	0.041	0.620	<0.001
Blue	bta-let-7b	*C14orf28*	97	-0.485	0.008	Milk yield	-0.509	0.006	0.424	0.029
Blue	bta-let-7b	*MXD1*	97	-0.457	0.014	Protein yield	-0.400	0.041	0.446	0.020
Blue	bta-let-7b	*PPP1R15B*	96	-0.420	0.025	Protein yield	-0.400	0.041	0.609	0.001
Blue	bta-let-7b	*QARS*	98	-0.386	0.041	Milk yield	-0.509	0.006	0.491	0.009
Blue	bta-let-7b	*SLC20A1*	96	-0.391	0.039	Milk yield	-0.509	0.006	0.502	0.007
Blue	bta-let-7b	*STX3*	97	-0.464	0.012	Protein yield	-0.400	0.041	0.531	0.004
Blue	bta-let-7b	*THTPA*	98	-0.431	0.021	Protein yield	-0.400	0.041	0.409	0.036
Blue	bta-let-7b	*TP53*	97	-0.413	0.028	Protein yield	-0.400	0.041	0.459	0.016
Blue	bta-miR-183	*CTDSP1*	98	-0.433	0.020	Protein yield	-0.452	0.018	0.443	0.021
Blue	bta-miR-183	*DGCR2*	99	-0.516	0.005	Protein yield	-0.452	0.018	0.442	0.021
Blue	bta-miR-183	*HLTF*	98	-0.428	0.022	Protein yield	-0.452	0.018	0.502	0.007
Blue	bta-miR-183	*HNRNPA1*	96	-0.479	0.009	Milk yield	-0.599	0.001	0.414	0.034
Blue	bta-miR-183	*ICA1*	99	-0.443	0.017	Protein yield	-0.452	0.018	0.548	0.003
Blue	bta-miR-183	*ILF2*	96	-0.373	0.050	C17:0	0.414	0.033	-0.400	0.041
Blue	bta-miR-183	*MAFF*	97	-0.427	0.022	Milk yield	-0.599	0.001	0.467	0.014
Blue	bta-miR-183	*MGME1*	98	-0.456	0.014	Milk yield	-0.599	0.001	0.399	0.042
Blue	bta-miR-183	*MTA1*	99	-0.475	0.010	Protein yield	-0.452	0.018	0.415	0.033
Blue	bta-miR-183	*PPP2R5C*	95	-0.446	0.017	Milk yield	-0.599	0.001	0.433	0.025
Blue	bta-miR-183	*RHBDD2*	95	-0.538	0.003	Protein percentage	0.547	0.003	-0.411	0.035
Blue	bta-miR-183	*RHPN2*	99	-0.407	0.031	Milk yield	-0.599	0.001	0.433	0.025
Blue	bta-miR-183	*SESN1*	97	-0.375	0.048	Protein yield	-0.452	0.018	0.396	0.043
Blue	bta-miR-183	*SFT2D1*	97	-0.500	0.006	C17:0	0.414	0.033	-0.425	0.028
Blue	bta-miR-183	*SPRY2*	99	-0.418	0.026	Protein yield	-0.452	0.018	0.488	0.009
Blue	bta-miR-183	*SRSF2*	98	-0.473	0.010	Protein yield	-0.452	0.018	0.617	0.001

Blue	bta-miR-183	UTP6	96	-0.432	0.021	protein yield	-0.452	0.018	0.545	0.003
Blue	bta-miR-183	*ZFAND5*	99	-0.427	0.022	protein yield	-0.452	0.018	0.430	0.026
Blue	bta-miR-2284b	*ACVR1*	96	-0.419	0.025	Milk yield	-0.442	0.021	0.416	0.032
Blue	bta-miR-2284b	*ARL15*	97	-0.443	0.017	protein yield	-0.429	0.026	0.427	0.027
Blue	bta-miR-2284b	*CCNT2*	96	-0.398	0.035	protein yield	-0.429	0.026	0.439	0.023
Blue	bta-miR-2284b	*CLIC2*	99	-0.416	0.027	protein yield	-0.429	0.026	0.610	0.001
Blue	bta-miR-2284b	*ERG*	95	-0.448	0.016	protein yield	-0.429	0.026	0.393	0.045
Blue	bta-miR-2284b	*FAM114A1*	96	-0.425	0.023	Milk yield	-0.442	0.021	0.459	0.016
Blue	bta-miR-2284b	*FAM8A1*	95	-0.386	0.042	protein yield	-0.429	0.026	0.414	0.033
Blue	bta-miR-2284b	*FAR1*	95	-0.387	0.041	Milk yield	-0.442	0.021	0.399	0.042
Blue	bta-miR-2284b	*IVNS1ABP*	95	-0.404	0.032	protein yield	-0.429	0.026	0.509	0.006
Blue	bta-miR-2284b	*LBR*	96	-0.510	0.005	protein yield	-0.429	0.026	0.462	0.015
Blue	bta-miR-2284b	*LIMA1*	97	-0.446	0.017	protein yield	-0.429	0.026	0.403	0.039
Blue	bta-miR-2284b	*NRROS*	96	-0.406	0.031	Milk yield	-0.442	0.021	0.480	0.011
Blue	bta-miR-2284b	*POLR2A*	99	-0.415	0.027	protein yield	-0.429	0.026	0.667	0.000
Blue	bta-miR-2284b	*RRN3*	96	-0.377	0.047	protein yield	-0.429	0.026	0.501	0.007
Blue	bta-miR-2284b	*SETD2*	97	-0.399	0.035	protein yield	-0.429	0.026	0.438	0.023
Blue	bta-miR-2284b	*SLC38A2*	95	-0.417	0.026	Milk yield	-0.442	0.021	0.526	0.004
Blue	bta-miR-2284b	*THAP2*	96	-0.385	0.042	protein yield	-0.429	0.026	0.445	0.020
Blue	bta-miR-2284b	*UBE4A*	96	-0.414	0.027	protein yield	-0.429	0.026	0.490	0.009
Blue	bta-miR-2284b	*ZDHHC17*	95	-0.384	0.043	protein yield	-0.429	0.026	0.426	0.028
Blue	bta-miR-2284b	*ZNF175*	97	-0.407	0.031	Milk yield	-0.442	0.021	0.433	0.025
Blue	bta-miR-23b-3p	*RBM4B*	95	-0.463	0.012	Milk yield	-0.390	0.047	0.549	0.003
Blue	bta-miR-30d	*CAMK2D*	95	-0.384	0.042	protein percentage	0.459	0.016	-0.433	0.025
Blue	bta-miR-409a	*ALG13*	99	-0.490	0.008	Milk yield	-0.553	0.002	0.462	0.015

Blue	bta-miR-409a	*GALNT5*	96	-0.482	0.009	Protein percentage	0.641	0.000	-0.616	0.001
Blue	bta-miR-409a	*RPL11*	98	-0.484	0.09	Fat percentage	0.387	0.049	-0.447	0.020
Blue	bta-miR-409a	*TMEM159*	99	-0.410	0.029	Fat percentage	0.387	0.049	-0.396	0.044
Blue	bta-miR-409a	*TRA2B*	97	-0.382	0.044	Milk yield	-0.553	0.002	0.598	0.001
Blue	bta-miR-6522	*FAM107B*	95	-0.384	0.043	Milk yield	-0.441	0.022	0.554	0.002
Blue	bta-miR-6522	*ZNF623*	95	-0.376	0.048	Milk yield	-0.441	0.022	0.550	0.003
Blue	bta-miR-96	*CHST1*	98	-0.373	0.050	Milk yield	-0.429	0.026	0.485	0.010
Blue	bta-miR-96	*EIF5*	96	-0.416	0.027	Milk yield	-0.429	0.026	0.494	0.009
Blue	bta-miR-96	*FARP1*	97	-0.466	0.012	Milk yield	-0.429	0.026	0.599	0.001
Blue	bta-miR-96	*GRHL2*	95	-0.398	0.035	Milk yield	-0.429	0.026	0.481	0.011
Blue	bta-miR-96	*LONP2*	97	-0.439	0.019	Fat percentage	0.613	0.001	-0.415	0.033
Blue	bta-miR-96	*PRKAR1A*	97	-0.375	0.049	Milk yield	-0.429	0.026	0.442	0.022
Blue	bta-miR-96	*SPIN1*	95	-0.392	0.038	Milk yield	-0.429	0.026	0.533	0.004
Blue	bta-miR-96	*SPROT*	97	-0.410	0.029	Milk yield	-0.429	0.026	0.535	0.004
Blue	bta-miR-96	*TP53*	95	-0.440	0.018	Milk yield	-0.429	0.026	0.427	0.027
Blue	bta-miR-96	*TRIB3*	97	-0.397	0.035	Fat percentage	0.613	0.001	-0.550	0.003
Blue	bta-miR-96	*ZCCHC3*	99	-0.453	0.015	Milk yield	-0.429	0.026	0.567	0.002
Brown	bta-miR-484	*CPPED1*	95	-0.413	0.028	C22:6n3	-0.402	0.040	0.600	0.001
Brown	bta-miR-484	*DOLPP1*	96	-0.393	0.037	C16:0	0.394	0.045	-0.396	0.043
Brown	bta-miR-484	*EIF1AD*	95	-0.470	0.011	Fat percentage	0.421	0.030	-0.432	0.025
Brown	bta-miR-484	*LY6E*	97	-0.420	0.025	C16:0	0.394	0.045	-0.394	0.045
Brown	bta-miR-484	*NUDT16*	96	-0.390	0.039	Fat percentage	0.421	0.030	-0.456	0.017
Brown	bta-miR-484	*QDPR*	99	-0.391	0.039	C16:0	0.394	0.045	-0.596	0.001
Turquoise	bta-miR-130a	*SBSPON*	96	-0.529	0.004	Protein percentage	-0.486	0.010	0.626	< 0.001
Turquoise	bta-miR-455-5p	*HPGD*	99	-0.384	0.043	Protein yield	0.491	0.009	-0.492	0.009

[1]Context++ score percentile from TargetScan prediction, [2]Correlation coefficient between miRNA and gene. [3]FDR (Benjamini-Hochberg corrected *P* -values) for Pearson correlation between miRNA and gene. [4]Correlation coefficient between miRNA and trait. [5]FDR for Pearson correlation between miRNA and trait. [6]Correlation coefficient between gene and trait. [7]FDR for Pearson correlation between gene and trait.

3.5 Discussion

Previously, we reported effects of diets rich in USFA on milk components and blood metabolites and on mRNA and miRNA expression in the bovine mammary gland (Ammah et al., 2018; Ibeagha-Awemu et al., 2016; Li et al., 2015). In the current study, we performed WGCNA of the miRNA data of the same animals and identified miRNA consensus modules (blue, brown, and turquoise) as well as miRNA–mRNA co-expressed pairs with potential effects on milk components, fatty acid phenotypes and blood metabolites. Nine miRNA members of the blue (bta-miR-30d, miR-96, miR-409a, miR-183, miR-99a-5p, miR-2285k, miR-652, miR-6522 and miR-374a), 5 of the brown (bta-let-7d, miR-885, miR-29b, miR-32 and miR-107) and 14 of the turquoise (bta-miR-16b, miR-130a, miR-142-5p, miR-218, miR-142-3p, miR-195, miR-19b, miR-455-3p, miR-15a, miR-424-5p, miR-106b, miR-155, miR-93 and miR-99a-3p) modules were previously reported as differentially expressed between lactation stages (Do et al., 2017). Additionally, 5 miRNAs (bta-miR-30d, miR-191, miR-151-5p, miR-99a-5p and let-7d), two miRNAs (bta-miR-26b and let-7g) and one miRNA (bta-miR-142-5p) in the blue, brown, and turquoise modules, respectively, were most abundant in milk fat, milk whey, milk cell and mammary gland tissues of lactating Holstein cows (Li et al., 2016) further supporting the influence of the blue, brown, and turquoise miRNA members on milk yield and components in this study. Previous studies have demonstrated the effects of diets rich in USFA on milk and blood components. Salehi et al. 2015, demonstrated the effects of diets rich in USFA on blood NEFA levels in dairy cows. Furthermore, increased blood plasma levels of NEFA in cows fed diets supplemented with flaxseed (linseed) and fish oil have been reported (Drackley, 1999; Gonthier et al., 2005; Petit et al.,2002). Similarly, TAG concentrations in serum increased when cows in early lactation received increasing levels of dietary fatty acids from canola oil (1% canola oil +1% fish oil, or 2% canola oil) (Vafa et al., 2012).

3.5.1 Blue Module miRNAs and Their Potential Roles

The blue module miRNAs were significantly correlated with milk and protein yields during the CP, milk yield, protein percent and lactose during treatment with SFO, protein percent and protein yield with LSO treatment. The miRNA expression data of these animals revealed that several of the blue module miRNAs were differentially expressed in response to dietary supplementation with SFO (bta-miR-96, miR-99a-5p, miR-199b, miR-16a, miR-484 and miR-99b) and LSO (bta-miR-885, miR-23b-3p and miR-99a-5p) (Li et al., 2015a), thus supporting their relationship to milk yield and milk components.

Many of the blue module miRNAs have been reported to play diverse roles in many biological processes. For example, human homologue of bta-miR-191 has been found to be dysregulated in different types of tumors in humans including colorectal (Xi et al., 2006), breast and prostate cancers (Volinia et al., 2006), while miR-199b is involved in acute myeloid leukemia (Garzon et al., 2008; Mi et al., 2007) and breast cancer metastasis (Huang et al., 2008; Shen et al., 2008; Fang et al., 2013). The implication of these miRNAs (bta-miR-191 and miR-199b) in the different types of cancers suggests roles in cellular functions in the mammary gland. The development of breast and prostate cancers is linked to cellular processes like cell death or apoptosis, therefore suggesting a link between these miRNAs and the milk phenotypes in this study. Some blue module miRNAs like bta-miR-183 and bta-miR-2284b correlated negatively with many candidate genes for protein yield (*CTDSP1*, *DGCR2*, *HLTF*, *ICA1*, *MTA1*, *SESN1*, *SPRY2*, *SRSF2*, *UTP6* and *ZFAND5)*, milk yield (*C14orf28*, *QARS*, *SLC20A1*, *HNRNPA1*, *MAFF*, *MGME1*, *PPP2R5* and *RHPN2*) and C17:0 fatty acid (*ILF2* and *SFT2D1*) (Figure 3.4) thus, suggesting potential roles in the regulation of these genes. In addition to the report of differential expression of some blue module miRNAs between lactation stages (Do et al., 2017), the importance of blue module miRNAs for milk yield and components is further supported by results of pathways enrichments of their target mRNAs. For instance, ErbB, MAPK, Wnt, TGF β and Hippo signaling pathways are involved in the regulation of mammary gland development and lactation processes (reviewed in Do and Ibeagha-Awemu, 2017). Furthermore, ErbB and TGF

β signaling pathways have been associated with lactation persistency in Holstein cow (Do et al., 2017). The p53 signaling pathway, the second most enriched pathway for the blue module, plays an important role in the prevention of cancer formation and hence acts as a tumor suppressor (Stegh,2012). The p53 gene has been nicknamed "guardian of the genome" due to its role in maintaining the stability of the genome (Surget et al., 2014). In addition, this pathway regulates proper cellular differentiation and development, and it is also important for tissues undergoing postnatal development (Jerry et al., 1999). Furthermore, inappropriate expression of the p53 signaling pathway within the mammary epithelium of transgenic mice caused apoptotic cell death of the alveolar epithelium of the mammary gland (Li et al., 1994). In this study, enrichment of p53 signaling pathway supports the significant correlation of the blue module miRNAs with milk yield, protein yield and lactose percent. Moreover, we also reported several enriched GO terms for the blue module filtered target mRNAs such as vesicle docking, negative regulation of transcription from RNA polymerase II promoter and proteasome−mediated ubiquitin-dependent protein catabolic process. However, it is not clear how these GO terms are linked to the studied phenotypes. We identified 22 important TF which could mediate the functions of miRNAs in the regulation of the target mRNAs or phenotypes (Table 3.5). *SMAD4*, *SP1* and *EGR1* were the top 3 TF enriched for the target mRNAs of the blue module miRNAs. *SMAD4* is a tumor suppressor gene and it is essential for transforming growth factor beta (TGFβ) signaling (Zhou et al., 1998), and plays important roles in cell differentiation, growth and apoptosis (Calva et al., 2011). Other important TF such as *STAT3* and *PPARG* are well known to regulate milk and milk fat synthesis (Chapman et al., 2002; Kang et al., 2015; Sargeant et al., 2014).

3.5.2 Brown Module miRNAs and Their Potential Roles

Bta-miR-484, a member of the brown module was previously reported as differentially expressed due to LSO and SFO treatments and it is also important in the regulation of lactation signaling (Li et al., 2015; Do et al., 2017) . Correlation analyses indicated that this miRNA negatively correlated with previously reported candidate genes for fat percentage (*EIF1AD* and *NUDT16*), C22:6n3 (*CPPED1*) and C16:0 (*LY6E*, *DOLPP1* and *QDPR*). The

LSO and SFO supplementation reduced fat percentage in the studied animals by 30.38% and 32.42%, respectively (Ibeagha-Awemu et al., 2016b) thus supporting the present observation. Additionally, bta-miR-484 has been observed to prevent cell proliferation and epithelial–mesenchymal transition process by targeting both *ZEB1* and *SMAD2*genes, thus functions like a tumor suppressor and may serve as a prospective biomarker for cervical cancer (Hu et al., 2017). Other members (bta-miR-26b and bta-miR-107) of the brown module have functions related to cellular processes (Li et al., 2016; Do et al., 2017) which are essential for lactation processes or milk synthesis. The human homologue of bta-miR-26b was shown to play a protective role in the etiology of breast cancer by promoting apoptosis through targeting *SLC7A11* (Liu et al., 2011) while bta-miR-107 is associated with mammary stem cell activities (Wicik et al., 2016).

The most significantly enriched biological process GO term in the brown and turquoise modules, GDP binding, is involved in cell proliferation, signal transduction, protein synthesis, and protein targeting (Kjeldgaard et al., 1996) (Table 3.3 Other enriched pathways in the brown module like MAPK signaling pathway plays an important part in numerous cellular processes such as apoptosis, proliferation and differentiation (Munshi and Ramesh, 2013), stress responses, and immune defense (Arthur and Ley, 2013; Dong et al., 2002) and have been noted to be important for mammary gland development and milk secretion in caprine (Hou et al., 2017).

The most enriched TF for the brown module was specific protein 1 (*SP1*) known to regulate the expression of numerous genes involved in cell proliferation, apoptosis and differentiation, and increase in its transcriptional activities is associated with tumorigenesis (Chang and Hung, 2012).

3.5.3 Turquoise Module miRNAs and Their Potential Role

The hub miRNA (bta-miR-16a) for the turquoise module has been reported to be differentially expressed in response to SFO treatment (Li et al., 2015). Bta-miR-16a being differentially regulated by SFO and also having the highest intra modular connectivity suggests involvement in the regulation of the traits (C14:0, C18:3n3 and 9, 11-CLA) that

were significantly correlated with the turquoise module in SFO treatment. Although a direct role for this miRNA in mammary gland functions has not been demonstrated, a previous study suggests its involvement in tumor suppression through inhibition of cell cycle progression (Jiang et al., 2016). Some turquoise module miRNAs like bta-miR-130a and bta-miR-142-5p have been linked to milk fat synthesis (Salehi et al., 2015; Yang et al., 2017) and disease parthenogenesis (Cobanoglu et al., 2006; Greenwood et al., 2009; Nawaz et al., 1999). Additionally, overexpression of bta-miR-130a affects cellular TAG synthesis in bovine mammary epithelial cells via regulation of *PPAR-γ* (Yang et al., 2017), fatty acid storage and glucose metabolism. Besides, some predicted target genes of miR-130a have been associated with neurodevelopmental disorders such as autism, schizophrenia and hereditary spastic paraplegia (Zhang et al., 2016). GO enrichment indicated that target mRNAs of turquoise module miRNAs participate in many different processes such as GTP binding, GDP binding, transcription coactivator activity, membrane organization, protein ubiquitination and regulation of apoptotic processes. Several KEGG pathways such as Ubiquitin mediated proteolysis, TGF-β signaling pathway and cell cycle, and tran TF scription factors such as *STAT3*, *SMAD4*, *SP1* and *EGR1* with roles in many different processes involving milk production and related traits (Do et al., 2017; Sargeant et al., 2014) were enriched for target mRNAs of turquoise module miRNAs. TGF-β signaling pathway and p53 signaling pathway were common pathways enriched by target mRNAs from all three modules and their roles in related traits have been discussed above. Ubiquitin mediated proteolysis, the most enriched pathway for turquoise module is important for protein degradation (Nawaz et al., 1999) and many processes including mediation of lactation signal in skeletal muscle of dairy cows (Greenwood et al., 2009). Top TF enriched for the target mRNAs of turquoise module were *SMAD4*, *SP1*, and *EGR1*. The early growth response protein 1 (*EGR1*) acts as a transcription regulator of target genes, hence play roles in the regulation of cell survival, proliferation, cell death response to growth factors, DNA damage, and ischemia (Weisz et al., 2004). A notable enriched TF was *STAT3* with known association with milk production (Cobanoglu et al., 2006), fertilization and embryonic survival rates (Khatib et al., 2009) in dairy cows.

3.5.4 Association Between miRNA and mRNA with Expressed Phenotypes

The most interesting part of this analysis is the different pathways linking miRNAs to the actual phenotypes through their target genes (Table 3.6 and Figure 3.4). Interestingly, different miRNAs that shared the same target genes regulated different traits. For instance, by targeting *TP53*, bta-let-7b might influence milk yield but also, bta-miR-96 might have an influence on protein percentage. Since many genes (mRNAs) and miRNAs influencing milk yield have been characterized such as *TP53*, *GRHL2,* bta-miR-183, bta-let-7b and bta-miR-96, we will not discuss about the network of miRNAs influencing milk yield. Meanwhile, for the first time, the link between some milk fatty acids, genes (mRNAs) and miRNAs have been reported. Interestingly, we observed negative correlation between bta-miR-484 and three different genes (*QDPR*, *LY6E* and *DOLPP1*) and positive correlation with C16:0 concentrations in milk. The roles of bta-miR-484 have been reported above, while it is not clear how these three genes are involved in the metabolism of C16:0. However, *DOLPP1* has a potential role in regulating subcutaneous fat (González-Calvo et al., 2017) while *LY6E* might play a role in the regulation of glycosylphosphatidylinositol. Also, *QDPR* is important for fat traits in pigs (Ponsuksili et al., 2011). Therefore, these genes might be interesting candidates for milk fat traits. The influence of bta-miR-183 on protein yield and protein percentage suggests that it can down regulate 8 different genes to influence protein yield. Nevertheless, these connections are based on correlations so it might not reflect true associations; therefore more studies are needed to validate the identified links and how they participate in mammary gland response to dietary USFA.

3.6 Conclusion

In this study, three consensus modules (blue, brown, and turquoise) composed of 70 (blue), 34 (brown) and 86 (turquoise) miRNA members were identified. We also demonstrated how miRNAs in these modules interacted with mRNAs to influence blood and milk phenotypes following dietary supplementation with USFA. Hub miRNAs for the blue, brown, and turquoise modules were bta-miR-30d, bta-miR-484 and bta-miR-16b, respectively.

Turquoise module had the most significant correlations with several traits including protein percentage in LSO treatment, protein yield, milk yield, C14:0, C18:3n3n and 9, 11-CLA in SFO treatment. The association of miRNA modules with milk and blood phenotypes has provided information about miRNA modules, hub miRNAs, GO terms, TF and pathways that are involved in the regulation of blood and milk parameters following dietary supplementation with diets rich in USFA. This study will contribute to the molecular understandings of the co-expression patterns of miRNAs, miRNA–mRNA, and regulatory activities in the bovine mammary gland following dietary supplementation with USFA.

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3.9 Supplementary table (supplementary tables is available in online version of published manuscript).

3.10 Supplementary figure

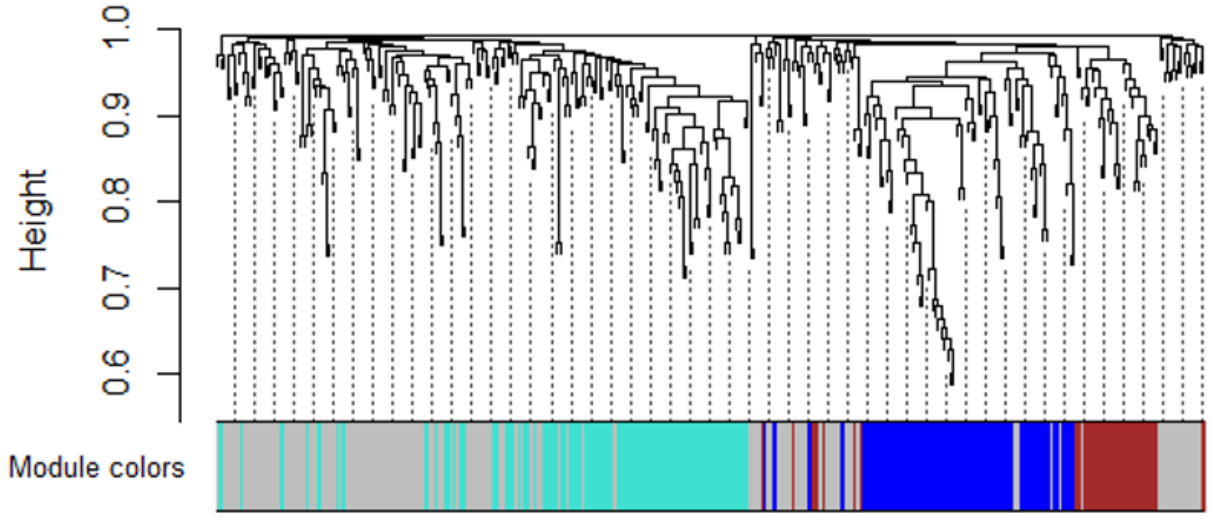

Figure 1. S1: The consensus gene dendrogram and module colors from weighted co-expression network analyses.

CHAPTER 4

Association between single nucleotide polymorphisms in lipogenic genes and *de novo* synthesized fatty acid profiles of Canadian Holstein cows

Article Description

In this manuscript, the association between SNP in genes involved in fatty acid and lipid biosynthesis pathways with *de novo* synthesized fatty acid profiles of Canadian Holstein cows was investigated. In the studies reported above, 5% LSO or 5% SFO depressed milk fat percentage by 30.38 % and 32.42 %, respectively. The proportion of C4:0, C6:0, C8:0 and C14:0 were decreased compared to long chain fatty acids (Ibeagha-Awemu et al. 2016). Genes in the fatty acid and lipid biosynthesis pathways were found to be differentially expressed in mammary gland tissues following dietary supplementation with 5% LSO or 5% SFO (Ibeagha-Awemu et al. 2016). We therefore investigated the association between DNA polymorphisms in genes in milk fatty acid synthesis and lipid biosynthesis pathways of Canadian Holstein cows . The manuscript will be submitted to the Canadian Journal of Animal Science.

Author Contributions

Conception and design of the study: Eveline Ibeagha-Awemu; Provided inputs on study design: Nathalie Bissonnette and Nicolas Gévry. Data collection: Adolf Ammah; Data analysis: Adolf Ammah, Do Duy; Interpretation of data: Adolf Ammah, Do Duy, Eveline Ibeagha-Awemu; Drafting of manuscript: Adolf Ammah and Do Duy; Critical revision of the manuscript: Eveline Ibeagha-Awemu; Revised and approved the final manuscript: All authors.

Targeted genotyping of polymorphisms in lipid and fatty acid biosynthesis pathway genes and association with *de novo* synthesized fatty acids in cow milk

Adolf A. Ammah[1, 2], Duy N. Do[1, 3], Nathalie Bissonnette[1], Nicolas Gévry[2] and Eveline M. Ibeagha-Awemu[1, 2, *]

[1]Agriculture and Agri-Food Canada, Sherbrooke Research and Development Centre, 2000 College Street, Sherbrooke, QC, J1M 0C8 Canada,

[2]Département de biologie, Université de Sherbrooke, 2500 Boulevard de l'Université, Sherbrooke, QC, J1K 2R1, Canada

[3]Department of Animal Science, McGill University, 21,111 Lakeshore Road, Ste. Anne de Bellevue, Quebec, H9X 3V9 Canada,

[*]**Corresponding author: Eveline.Ibeagha-Awemu@AGR.GC.CA**

4.1 Abstract

Our previous study showed that supplemental feeding of 5% LSO or 5% SFO depressed milk fat percentage by 30.38% and 32.42%, respectively with a higher impact on *de novo* synthesized fatty acids. Transcriptome mRNA analysis of mammary gland biopsies from these cows revealed 18 differentially expressed genes of the fatty acid and lipid biosynthesis pathways. In this study, we further investigated the association between SNP in these genes (18) of the fatty acid and lipid biosynthesis pathways with *de novo* synthesized fatty acid profiles in milk from a larger population of Canadian Holstein cows. Five hundred and seventy-seven (n = 577) milk samples collected from about twenty dairy herds in Quebec were analyzed for their *de novo* synthesized fatty acid profiles. Association between 40 potentially functional SNPs (13 missense mutations, 9 synonymous mutations, 9 mutations in the 3'UTR and 9 mutations in the 5' UTR) in genes involved in *de novo* synthesized fatty acids were determined. Analysis indicated that seven SNPs (rs380146565, rs133629324, rs43167426, rs210512360, rs133629324, rs42199236, rs137182814) in *AGPAT9, STAT5A, SCP2, OLR1* and *CPT1A* genes were associated ($P = 0.05$) with *de novo* synthesized fatty acids. A significant relationship ($P = 0.007$) between rs380146565, a mutation in the 3′ UTR within the *AGPAT9* gene and C4:0 was revealed; in which case, genotype GG was associated with the highest increase in C4:0. Allele substitution at this locus (allele G substituted for T) indicated that allele T reduced ($P = 0.035$) C4:0 content by 0.222 ± 0.099 g/100g of fat. Similarly, rs137182814, synonymous mutation within the *STAT5A* gene was significantly ($P = 0.050$) associated with C16:0 fatty acid while genotype GG was associated with the highest increase. Meanwhile, allele C substituted for G revealed that G increased ($P = 0.050$) milk C16:0 by 0.754 ± 0.367 g/100g of fat. The identified associated genetic variations could facilitate breeding for decreased milk *de novo* synthesized fatty acids.

Keywords: Single nucleotide polymorphism, *de novo* synthesized fatty acid, lipid biosynthesis pathway, fatty acid biosynthesis pathway, dairy cow, milk.

4.1 Résumé

Notre étude précédente a montré que l'alimentation complémentaire de 5% d'huile de lin (LSO) ou de 5% d'huile de carthame (SFO) a réduit le taux de graisse du lait de 30,38% et 32,42% respectivement, avec un effet plus accentué sur les acides gras synthétisés de novo, tandis que l'analyse du mRNA transcriptome des biopsies de glande mammaire des mêmes vaches a révélé dix-huit gènes exprimés de manière différente des voies de biosynthèse des acides gras et des lipides. Dans cette étude, nous avons étudié en profondeur l'association entre les polymorphismes a nucléotidiques simples (SNP) dans ces gènes (18) des voies de biosynthèse des acides gras et des lipides avec les profils d'acides gras synthétisés de novo dans une plus grande population de vaches Holstein canadiennes. Cinq cent soixante-dix-sept échantillons de lait prélevés dans une vingtaine de troupeaux laitiers du Québec ont été analysés pour leurs profils d'acides gras synthétisés de novo. L'association entre 40 SNPs potentiellement fonctionnels (13 mutations faux-sens, 9 mutations synonymes, 9 mutations dans les 3'UTR et 9 mutations dans les 5' UTR) dans les gènes et les acides gras synthétisés de novo ont été déterminés statistiquement. L'analyse d'association a indiqué que sept SNPs (rs38014656565, rs133629324, rs43167426, rs210512360, rs133629324, rs42199236, rs137182814) dans les gènes *AGPAT9, STAT5A, SCP2, OLR1* et *CPT1A* étaient associés de manière significative (P = 0,05) avec les acides gras de novo synthétisés. Une relation significative (P = 0,007) entre rs38014656565, une mutation dans l'UTR 31 au sein du gène *AGPAT9* et C4:0 a été révélée ; dans ce cas, le génotype GG a été associé à l'augmentation la plus élevée en C4:0. La substitution d'allèle à ce locus (allèle G substitué à T) indique que l'allèle T a réduit (P = 0,035) la quantité de C4:0 de 0,222 ± 0,099. Pareillement, rs137182814, mutation synonyme au sein du gène *STAT5A* était significativement (P = 0,050) associé à l'acide gras C16:0 et le génotype GG était associé à l'augmentation la plus élevée. Par ailleurs, l'allèle C substitué à G a révélé que G a augmenté (P = 0,050) le lait C16:0 de 0,754 ± 0,367. Les variations génétiques associées identifiées pourraient faciliter l'élevage pour une diminution des acides gras synthétisés du lait de novo.

Mots-clés: Polymorphisme a nucléotide simple, acide gras synthétisé de novo, voie de biosynthèse des lipides, voie de biosynthèse des acides gras, voie de biosynthèse des acides gras, vache laitière, lait.

4.2 Introduction

In a previous study we showed that supplemental feeding of 5% LSO or 5% SFO depressed milk fat percentage by 30.38% and 32.42%, respectively and in particular *de novo* synthesized fatty acids were most affected (Ibeagha-Awemu et al. 2016). Furthermore, transcriptome mRNA analysis of mammary gland biopsies from the same cows revealed eighteen differentially expressed genes of the fatty acid and lipid biosynthesis pathways in response to the lipid supplements (Ibeagha-Awemu et al. 2016). Fatty acid in milk are either taken up from blood circulation or are synthesized *de novo* in the mammary gland. Short and medium chain length fatty acids (C4:0 to C14:0) are almost exclusively (~95%) synthesized *de novo* in the mammary gland, LCFA (>16 carbons) are mostly up taken from circulation while about 50% of C16:0 is synthesized *de novo* and the other 50% is up taken from circulating blood (reviewed in Bauman and Griinari, 2003, Shingfield et al., 2010, Chilliard et al., 2000). Additionally, fatty acids mostly synthesized *de novo* (C4:0 to C16:0) in the mammary gland occur in relatively high concentrations in milk and show moderately high heritability (range from 0.10 to 0.50) (Bastin et al.,2013; Lopez-Villalobos et al., 2014; Olsen et al., 2017) making them suitable for genetic analysis (Knutsen et al., 2018) and targets for genetic improvement.

Dietary modifications through feeding trials have clearly shown that it is an effective way to rapidly modify milk fat composition (Shingfield et al., 2013). However, feeding effects are totally revocable when feeding conditions change (Shingfield et al., 2013) coupled with low transformation efficiency of dietary fatty acids into milk unsaturated fatty acids (USFA) due to ruminal biohydrogenation of dietary fatty acids in the rumen of the animal (Doreau and Chilliard, 1997).

Dairy producers are therefore looking for different means to improve the fatty acid profile of milk through various techniques including dietary and genetics strategies (Jensen, 2002; Nafikov et al., 2013a, b). Genetic improvement can slowly induce minor but permanent trait variations and have shown heritable variations in the fatty acid profile of bovine milk (Bastin et al., 2013; Krag et al., 2013; Pegolo et al., 2016). Moreover, selective breeding can be an

effective technique to modify the nutritional quality of milk (Schennink et al., 2007; Soyeurt et al., 2008). Candidate gene studies have shown that SNP in some key lipogenic genes like diacylglycerol o-acyltransferase 1 (*DGAT1*), sterol regulatory element binding protein-1 (*SREBP1*), and stearoyl-CoA desaturase *(SCD)* influenced milk fat composition and explained part of the genetic variation of milk fat unsaturation indices seen in some dairy breeds like Holsteins (Schennink et al., 2007; Schennink et al., 2008; Rincon et al., 2012) and Brown Swiss cows (Mele et al., 2007; Conte et al., 2010). An association study found that SNPs in fatty acid synthase (*FASN)*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A)*, ATP binding cassette subfamily G member 2 (*ABCG2),* and insulin-like growth factor 1 (*IGF1)* genes were mostly associated with medium-chain and long-chain fatty acid, specifically *FASN* with C10:0, C12:0 and C14:0 (Li et al., 2016).

Through genome-based selection methods, it is possible to improve our knowledge of the pathways that are invloved in bovine milk fat synthesis, classify genes, and genetic polymorphisms that are associated with variations in milk fatty acid content (MacLeod, et al., 2016) and to optimally balance individual fatty acids in milk in order to meet up with consumer demands for high quality and healthy foods (Knutsen et al. 2018). The potential targets for this purpose are the short and medium chain fatty acids (C4:0 to C14:0) because they are synthesized *de novo* in the bovine mammary gland, in contrast to most long chain milk fatty acids and about half of C16:0 which are largely derived from the cow's diet (Bauman and Griinari, 2003).

Therefore, the aim of this study was to determine the relationship between SNPs in 18 genes of the fatty acid and lipid biosynthesis pathways reported as differentially expressed in our recent study (Ibeagha-Awemu et al., 2016) and the level of *de novo* synthesized milk fatty acids within a larger population of Canadian Holstein cows.

4.3 Materials and Methods`

4.3.1 Animal Sampling

Five hundred and seventy-seven milk samples (100 ml/cow) were collected from 18 dairy herds in Quebec with the assistance of Valacta Inc. (Ste-Anne-de-Bellevue, QC, Canada, www.valacta.com). Fat and somatic cell fractions were separated by centrifugation at 4500 x g for 20 min at 4 °C. The fat layer was used for the determination of fatty acid profiles while DNA was isolated from somatic cells and used for genotyping. Procedures for animal care and use were according to the national codes of practice for the care and handling of farm animals (Canadian Council on Animal Care, 2009, http://www.ccac.ca/en_/standards/guidelines) and approved by the animal care and ethics committee of Agriculture and Agri-Food Canada.

4.3.2 Genomic DNA isolation and purification from milk somatic cells

After centrifugation of the milk, the uppermost portion (fat) was extracted using a clean spatula and transferred in a 20 ml disposable scintillation vial. The middle layer (whey) was decanted and the lowermost part (cells) was re-suspended in 1 mL of 1 x PBS, centrifuged at 4,500 x *g* for 20 min at 4ºC. The supernatant was decanted and the milk cells at the bottom of the tube were stored at -20ºC. Genomic DNA was isolated from cells using NucleoSpin Blood Quick Pure Kit (Macherey-Nagel, Duren, Germany) adapted for milk cells. Briefly, thawed cells were washed three times by addition of 800 µl 1× PBS solution, centrifuged at 15,000 x *g* for 5 min at 4ºC and about 800 µl of the supernatant removed. After the final centrifugation step, the cells were re-suspended in 25µl of proteinase K (10 mg/mL) and 200 µl of buffer B3 was added and the mixture was vortexed vigorously for 10-20 seconds. The samples were then incubated at 70ºC for 10-15 min. The aqueous phase was transferred to a 2 ml tube and 210 µl ethanol (100%) was added, vortexed briefly, and transferred to the DNA column of NucleoSpin Blood QuickPure Kit. It was centrifuged at 11,000 x *g* for 1 min. for binding DNA. 500 µl wash buffer was added to the column and centrifuged at 11,000 x *g* for 1 min. and this washing step was repeated twice. The silica membrane was dried by centrifugation at 11,000 x *g* for 1 min.

Deoxyribonucleic acid was eluted using 150 µl pre-heated (70 ºC) elution buffer as recommended and stored at -20°C until used for genotyping.

4.3.3 Fatty acid analysis

Milk fat was used to prepare fatty acid methyl esters according to O'Fallon et al. (2007). Briefly, milk fat was permeabilized and hydrolyzed for 1.5 h at 55ºC in 1 N KOH in methanol containing C13:0 as the internal standard. KOH was neutralised, and the mixture was methylated by H_2SO_4 catalysis for 1.5 h at 55°C. Three ml of hexane was added to the single screw-cap Pyrex reaction tube and mixed by vortexing followed by centrifugation (3750 x *g* for 5 min). The hexane layer, which contain the FAME, was transferred into a gas chromatography vial and stored at -20ºC until analyzed.

Fatty acid methyl esters were analyzed using a Hewlett Packard 6890N gas chromatographic system (Agilent Technology, Wilmington, DE, USA) equipped with a flame ionization detector and an auto sampler (Hewlett Packard, Avondale, PA, USA). The gas chromatographic capillary column used was SLB-IL111 (100 m x 0.25 mm, 0.2 µm in thickness, Supelco, Bellefonte, PA, USA). The gas carrier was hydrogen at 1 mL/min constant flow with a linear velocity of 26 cm/s. The column temperature was set at a start temperature of 40°C for 1 min., then ramped at 8°C /min. to 170°C and held for 1min., then 4°C /min. to 195°C and held for 2min., and finally, at 2°C /min. to 210°C and then held for 15 min. The injection port and detector temperatures were set at 250^0C. The split ratio was set to 100:1 and the injection volume was 1µl. Individual FAME peaks were identified by comparison of retention times with FAME standards (GLC No. 463 and No. UC-59-M, Nu-Chek Prep Inc., Elysian, MN, USA). Agilent Technologies Chemstation B.04.03 software was used for data analysis.

4.3.4 SNP mining from RNA-Seq data

Single nucleotide polymorphisms in 18 differentially expressed genes (Table 4.1) related in fatty acid and lipid biosynthesis pathways from our previous study (Ibeagha-Awemu et al., 2016) were identified using the integrative genome viewer (IGV) software (http://software.broadinstitute.org/software/igv/). The genes are insulin-induced gene 1[*INSIG1*], signal transducer and activator of transcription 5A [*STAT5A*] and 5B [*STAT5B],* sterol regulatory element binding transcription factor 1 [*SREBF1*] and tribbles pseudokinase 3 [*TRIB3]*), TAG synthesis (glycerol-3-phosphate acyltransferase 3 [*AGPAT9*], diazepam binding inhibitor [*DBI*] and acyl-CoA binding protein [*ACBP*]), fatty acid metabolism (acyl-CoA dehydrogenase family member 8 [*ACAD8]*, carnitine palmitoyltransferase 1A [*CPT1A]*) and energy production (uncoupling protein 2 [*UCP2*]) (Table 4.1).

4.3.5 SNP genotyping

Forty potential functional SNPs (Table 4.2) identified in the 18 genes (Table 4.1) were genotyped in 577 individual DNA samples by the method of Sequenum iPLEX Gold Technology on a MassARRAY platform (Sequenom Inc. San Diego, CA, USA) by Genome Quebec and McGill University Innovation Centre (https://genomequebec.mcgill.ca/).

4.3.6 Statistical methods

The animal model was used to test associations between SNPs and phenotypes. The pedigree of cows was traced back to 5 generations and included 1,160 sires and 3,783 dam records. The following linear mixed model which fitted each SNP at a time was used:

$y = Xb + mg + Z_a a + Z_{HYS} HYS + e,$

where y = vector of fatty acids (7 phenotypes), b = vector of fixed effects (parity, age at calving and stage of lactation), X = incidence matrix for fixed effects, m is a vector with genotypic indicators (-1, 0, or 1) associating records to the marker effect, g is a scalar of the associated additive effect of the SNP, a = vector of random animal effects, HYS = vector of random HYS

effects, Z_a = incidence matrix for random animal effects, Z_{hys} = incidence matrix for random HYS effects and e = random residual effects. The animal, HYS and residual terms were assumed to be independent and distributed as follows: a ~ N(0, $A\sigma_a^2$), HYS ~ N(0, $I\sigma_{HYS}^2$) and e ~ N(0, $I\sigma_e^2$), where σ^2_a , σ^2_{hys} and σ^2_e are variances of random animal genetic effect, random HYS effect and random residual error effect, respectively, and A is the numerator relationship matrix across all animals inferred from the pedigree, and I is the identity matrix.

All association analyses were performed using the DMU software (http://dmu.agrsci.dk; (Madsen et al., 2006). The effects of SNPs on the phenotypes were tested using a Wald test against a null hypothesis of g = 0 (SNP has no effect). The corrected *P*-values (p.BH, Benjamini and Hochberg) were computed following the method of controlling false discovery rate (Benjamini and Hochberg, 1995), and p.BH < 0.05 were considered as significant value for the trait.

4.4 Results

4.4.1 SNPs and functional classification

Forty potential functional SNPs identified from RNA-Seq data of 18 genes included 13 missense (non-synonymous, lead to an amino acid change) variants, 9 synonymous mutations (no change in the affected amino acids), 9 mutations in the 3'UTR and 9 mutations in the 5'UTR (Table 4.2). Four SNPs (rs133974976, rs476649393, rs41784335, and rs42720309) did not amplify in any sample and were not further considered (Table 4.2). Allele and genotype frequencies in the studied herds (18 herds) are shown in Supplementary Table S4.1.

Table 4.1 Selected genes and their functions

Gene symbol (name)	Chr.	Acession number	Gene function
INSIG1 (insulin-induced gene 1)	4	NM_001077909.1	Encodes a protein that blocks the proteolytic activation of sterol regulatory element binding proteins, which are transcription factors that trigger genes that regulates fatty acid, cholesterol and glucose metabolism.
UCP2 (uncoupling proteins 2)	15	NP_001028783	UCP are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis. As a result, energy is dissipated in the form of heat.
FBP2 (fructose-bisphosphatase 2)	8	NM_001046164	Catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate in the presence of divalent cations and probably participates in glycogen synthesis from carbohydrate precursors, such as lactate.
OLR1 (oxidized low-density lipoprotein (lectin-like) receptor 1)	5	NC_005027.1	Receptor that mediates the recognition, internalization and degradation of oxidatively modified low-density lipoprotein (LDL) by vascular endothelial cells.
KCNMA1 (potassium channel, calcium activated large conductance subfamily M alpha, member 1)	28	NM_174680.2	Potassium channel activated by both membrane depolarization and increase in cytosolic Ca^{2+} that mediates export of K^{+}.
STAT5A (signal transducer and activator of transcription 5A)	19	NM_001012673	Conveys a signal across a cell to trigger a change in cell function or state
STAT5B (signal transducer and activator of transcription 5B)	19	NM_174617.4	Signal transduction and activation of transcription and also mediates cellular responses to the cytokine KITLG/SCF and other growth factors.
PIGZ (phosphatidylinositol glycan anchor biosynthesis class Z)	1	XM_024994883.1	Encodes a protein that is localized to the endoplasmic reticulum and is involved in GPI (glycosylphosphatidylinositol) anchor biosynthesis.
ACAD8 (Acyl-CoA dehydrogenase family member 8)	15	NM_001075551	This gene encodes a member of the acyl-CoA dehydrogenase family of enzymes that catalyze the dehydrogenation of acyl-CoA derivatives in the metabolism of fatty acids or branch chained amino acids.
CPT1A (carnitine palmitoyltransferase 1A)	29	NM_001304989.2	Rate-limiting enzyme for fatty acid β-oxidation,
AGPAT9 (glycerol-3-phosphate acyltransferase 3)	6	NM_001192514.**3**	Catalyze the initial step of *de novo* TAG synthesis by converting glycerol-3-phosphate to lysophosphatidic acid hence playing a key role in the regulation of cellular TAG and phospholipid levels.
IGSF9B (immunoglobulin superfamily member 9B)	29	XM_024987414.1	Kinase binding
SCP2 (sterol carrier protein 2)	3	NM_001033990.3	Mediates in vitro transfer of all common phospholipids, cholesterol and gangliosides between membranes.
DB1 (diazepam binding inhibitor (acyl-CoA binding protein)	2	NM_001113321	Intracellular carrier of acyl-CoA esters
TRIB3 (tribbles pseudokinase 3)	13	NM_001076103.1	Disrupts insulin signaling by binding directly to Akt kinases and blocking their activation.

PIK3CG (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma)	4	NM_001102125.2	Phosphoinositide-3-kinase (PI3K) that phosphorylates PtdIns (4,5) P2 (Phosphatidylinositol 4,5-bisphosphate) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3). Involve in transferase activity, transferring phosphorus-containing groups and binding
CLDN7 (claudin 7)	19	NM_001040519.2	Plays a major role in tight junction specific alliteration of the intracellular space
SREBF1 (sterol regulatory element binding transcription factor 1)	19	NM_001113302.1	They are known to regulate the transcription of lipogenic genes.

Table 4.2 Characteristics of single nucleotide polymorphisms used in the marker-trait association study

	Gene	SNP	rs number	Chromosome	*Position	Functional impact (Residue change)
Passed markers[1]						
1	*INSIG1*	G>A	rs110728454	4	117918953	3[1] UTR variant (NA)
2	*INSIG1*	C>G	rs42305262	4	117919243	3[1] UTR variant (NA)
3	*INSIG1*	G>A	rs41256951	4	117919734	3[1] UTR variant (NA)
4	*UCP2*	G>C	rs381477406	15	54201608	5[1] UTR variant (NA)
5	*UCP2*	G>T	rs135589290	15	54201612	5[1] UTR variant (NA)
6	*UCP2*	G>A	rs109407261	15	54201699	5[1] UTR variant (NA)
7	*UCP2*	C>T	rs383439735	15	54202579	5[1] UTR variant (NA)
8	*UCP2*	A>G	rs378396093	15	54202602	5[1] UTR variant (NA)
9	*FBP2*	A>G	rs133155622	8	82414193	Synonymous, (Ala166Ala., GCA >GCG)
10	*FBP2*	G>A	rs132777464	8	82433356	Synonymous (Lys108Lys, AAG >AAA)
11	*OLR1*	A>C	rs133629324	5	100247877	Missense (Lys65Gln, AAA > CAA)
12	*KCNMAI*	C>T	rs134429681	28	33069138	Synonymous (Tyr343Tyr, TAC > TAT)
13	*STAT5A*	C>T	rs380833204	19	43045167	Synonymous (Asn264Asn, AAC > AAT)
14	*STAT5A*	C>T	rs378205101	19	43045179	Synonymous (Pro268Pro, CCC > CCT)
15	*STAT5A*	T>C	rs448701112	19	43045675	Synonymous (Ile285Ile, ATT > ATC)
16	*STAT5A*	C>G	rs137182814	19	43045807	Synonymous (Thr329Thr, ACC > ACG)
17	*STAT5B*	G>A	rs133665517	19	42984431	Missense (Gly40Ser, GGC > AGC)
18	*PIGZ*	A>T	rs134024539	1	72028786	5[1]UTR variant (NA)
19	*PIGZ*	C>T	rs382020465	1	72028854	5[1]UTR variant (NA)
20	*ACAD8*	A>G	rs41784332	15	84874316	3[1] UTR variant (NA)
21	*ACAD8*	C>G	rs41784334	15	84874475	3[1] UTR variant (NA)
22	*CPT1A*	C>T	rs42199236	29	46859241	Synonymous (Asn88Asn, AAC > AAT)
23	*AGPAT9*	C>T	rs43167426	6	100112922	Synonymous (Thr162Thr, ACC > ACT)
24	*AGPAT9*	G>T	rs380146565	6	100140476	3[1] UTR variant (NA)
25	*AGPAT9*	C>T	rs482502090	6	100141281	3[1] UTR variant (NA)
26	*IGSF9B*	G>C	rs876383654	29	33930232	Missense(Gly1085Ala,GGC>GCC)
27	*IGSF9B*	C>A	rs876095260	29	33920144	Missense (His351Asn,CAC > AAC)
28	*SCP2*	A>G	rs210512360	3	93885052	Missense, (Ile393Val, ATC> GTC)

29	*SCP2*	A>G	rs211310529	3	93952047	Missense (Ile128Val, ATT > GTT)
30	*DB1*	C>T	rs210945785	2	71561198	5[1]UTR variant (NA)
31	*TRIB3*	C>T	rs207783172	13	61183469	Missense (Pr**o**60Leu**,** CCG > CTG)
32	*TRIB3*	C>T	rs210704544	13	61187817	5[1]UTR variant (NA)
33	*INSIG1*	A>G	rs111011933	4	117916210	Missense (His270Arg**,** CAC > CGC)
34	*ACAD8*	T>G	rs470242182	15	84865538	Missense (Val90Ala, GTG > GCG)
35	*PIK3CG*	T>A	rs435689622	4	48270678	Missense (318Val **>**Glu**,** GTG > GAG)
36	*INSIG1*	C>G	rs132633746	4	117916228	Missense (Ala276Gly**,** GCA > G**G**A)
Failed markers[2]						
37	*INSIG1*	C>A	rs133974976	4	117918581	3[1] UTR variant(NA)
38	*SREBF1*	A>C	rs476649393	19	35247787	Missense (Thr801Pro, ACT > CCT)
39	*ACAD8*	C>T	rs41784335	15	84874645	3[1] UTR variant (NA)
40	*SCP2*	A>G	rs42720309	3	93936779	missense(Ile143Val, ATT > GTT)

Passed markers[1], markers that amplified in atleast one sample
Failed markers[2], markers that never amplified in any sample

4.4.2 Marker-trait association results

Marker trait association results are shown in Table 4.3 and results of significant allele substitution effects are shown in Table 4.4. Seven of the 36 SNPs investigated showed significant associations ($P < 0.05$) with milk *de novo* synthesized fatty acid contents **(**Table 4.3).

A significant relationship ($P = 0.007$) between rs380146565, a mutation in the 3' UTR of the *AGPAT9* gene and C4:0 was revealed; in which case, genotype GG was associated with the highest increase in C4:0. Allele substitution at this locus (allele G substituted for T) indicated that allele T reduced ($P = 0.035$) C4:0 content by 0.222 ± 0.099 g/100 g of total fat (Table 4.4). Similarly, a significant association ($P = 0.035$) was recorded between rs133629324, a missense mutation within the *STAT5A* gene and C6:0 and genotype GT was associated with the highest increase. Allele substitution at this locus (allele G substituted for T) showed that allele T reduced ($P = 0.010$) milk C6:0 by 0.188 ± 0.068 g/100 g of total fat (Table 4.4). Significant ($P = 0.049$) genetic association was recorded between rs210512360 (a missense mutation within the *SCP2* gene) and C11:0. Genotype CC at this locus was associated with the highest increase in C11:0. This is supported by allele substitution analysis whereby substituting allele C for T reduced ($P = 0.040$) C11:0 by 0.054 ± 0.025 g/100 g of total fat (Table 4.4). Additionally, rs137182814, a synonymous mutation within the *STAT5A* gene was significantly ($P = 0.050$) associated with

C16:0 fatty acid and genotype GG was associated with the highest increase. Allele substitution at this locus (allele C substituted for G) showed that allele G increased (P = 0.050) C16:0 by 0.754 ± 0.367 g/100 g of total fat (Table 4.4). Likewise, the synonymous mutation (rs43167426) in *AGPAT9* gene had a significant (P = 0.039) association with C8:0 and the genotype CC was associated with the highest increase. Allele substitution at this locus (allele C substituted for T) showed that allele T reduced significantly (P = 0.008) C8:0 by 0.219 ± 0.076 g/100 g of total fat (Table 4.4).

Table 4.3 Markers associated with *de novo* synthesized fatty acids (g/100 g of total fat) in Canadian Holstein cows

Fatty acid	SNP	Functional impact	Gene	Means of genotypes									*P*-value
				CC	CT	TT	AA	AG	AT	AC	GG	CG	GT	
C4:0	rs380146565	3[1] UTR variant	*AGPAT9*			2.774[ab]	-	-	-	-	2.845[b]	-	2.522[a]	0.007
C6:0	rs43167426	Synonymous (Thr162Thr, ACC > ACT)	*AGPAT9*	2.249	2.354	2.437								0.526
	rs134429681	Synonymous (Tyr343Tyr, TAC > TAT)	*KCNMAI*	-	-	-	-	2.330	-	-	2.386	-	-	0.780
	rs133629324	Missense (Lys65Gln, AAA > CAA)	*STAT5A*	-	-	2.365[a]	-	-	-	-	2.426[ab]	-	2.538[b]	0.035
	rs382020465	5[1]UTR variant	*PIGZ*	2.408	2.459	2.665	-	-	-	-	-	-	-	0.499
C8:0	rs43167426	Synonymous (Thr162Thr, ACC > ACT)	*AGPAT9*	1.977[b]	1.252[a]	1.070[a]	-	-	-	-	-	-	-	0.039
C11:0	rs210512360	Missense, (Ile393Val, ATC> GTC)	*SCP2*	0.526[b]	0.401[ab]	0.357[a]	-	-	-	-	-	-	-	0.049
C12:0	rs134024539	5[1]UTR variant	*PIGZ*	-	-	1.717	1.894	-	1.809	-	-	-	-	0.084
C15:0	rs133629324	Missense (Lys65Gln, AAA > CAA)	*OLR1*	-	-	0.382[a]	-	-	-	-	0.247[ab]	-	0.530[b]	0.014
C16:0	rs42199236	Synonymous (Asn88Asn, AAC > AAT)	*CPT1A*	25.630[b]	24.136[b]	22.749[a]	-	-	-	-	-	-	-	0.002
	rs137182814	Synonymous (Thr329Thr, ACC > ACG)	*STAT5A*	24.153[a]	-	-	-	-	-	-	25.648[b]	24.722[ab]	-	0.050

[a, b, c]For each fatty acid, genotype means (g/100 g of total fat) with different superscripts differ significantly ($P < 0.05$).

Table 4.4 Results of significant allele substitution effects

Fatty acid	Common name	SNP	Effect allele	Gene	Estimate ± SE	*P*-value	*P*-adjust
C4:0	Butyric acid	rs380146565	T	*AGPAT9*	-0.222±0.099	0.035	0.453312
C6:0	Caproic acid	rs133629324	T	*STAT5A*	-0.188±0.068	0.010	0.108807
C8:0	Caprylic acid	rs43167426	T	*AGPAT9*	-0.219±0.076	0.008	0.239969
C11:0	Undecanoic acid	rs210512360	T	*SCP2*	-0.054±0.025	0.040	0.990926
C15:0	Myristic acid	rs133629324	T	*OLR1*	-0.187± 0.042	1.0E-3	0.004688
C16:0	Palmitic acid	rs42199236	T	*CPT1A*	-1.419±0.393	0.001	0.039049
		rs137182814	C	*STAT5A*	0.754±0.367	0.050	0.483746

4.5 Discussion

This study examined associations between SNPs in 18 genes of the fatty acid and lipid biosynthesis pathways previously shown to be differentially expressed in the bovine mammary gland following dietary supplementation of cow's diets with 5% SFO or 5% LSO (Ibeagha-Awemu et al. 2016) with milk *de novo* synthesized fatty acids in Canadian Holstein cows. The 18 genes (*INSIG1, UCP2, FBP2, OLR1, KCNMAI, STAT5A, STAT5B, PIGZ, ACAD8, CPT1A, AGPAT9, IGSF9B, SCP2, DB1, TRIB3, PIK3CG, CLDN7* and *SREBF1*) were studied based on their roles in the regulation of milk fatty acid and lipid metabolism (Ibeagha-Awemu et al. 2016; Bionaz and Loor, 2008; Shingfield et al., 2010).

From the intestines, C2 (acetate) and C4 (butyrate) which are precursors for the synthesis of C4:0 to C14:0 fatty acids are transported through the blood stream to the mammary gland where acetate and acetoacetate are changed to acetyl-CoA, then to malonyl-CoA which with butyryl-CoA are used as precursors for cytosolic *de novo*-synthesis (Knutsen et al. 2018). The cyclical elongation process in which there is extension of carbon chain from C4:0 to C16:0 also generates intermediate products. Newly produced fatty acids in the mammary epithelial cells are transported from the cytosol to the endoplasmic reticulum where they are connected to a glycerol

3-phosphate backbone to form TAG. The last stage of this synthesis is the secretion of *de novo* synthesized fatty acids into milk in the form of milk fat globules (Knutsen et al. 2018).

In this study, seven SNPs in 5 genes (*AGPAT9, STAT5A, SCP2, OLR1* and *CPT1A)* associated significantly ($P \leq 0.05$) with C4:0 (rs380146565), C6:0 (rs133629324), C8:0 (rs43167426), C11:0 (rs210512360), C15:0 (rs133629324) and C16:0 (rs137182814 and rs42199236). To improve wellness, a reduction in the overall consumption of SFAs, trans-fatty acids and cholesterol has been put forth while emphasis has been placed on the need to increase intake of USFA (Griel and Kris-Etherton, 2006; Kris-Etherton et al., 2007; Salter, 2011). Saturated fatty acids, mainly C12:0, C14:0 and C16:0 are considered to produce negative health effects when consumed in excess (Mensink et al., 1994).

Three synonymous mutations in this study were significantly associated ($P \leq 0.05$) with C8:0 (rs43167426) and C16:0 (rs137182814 and rs42199236). Synonymous mutations have normally been regarded as silent mutations with no effect on protein structure and function. However, it has been proven that silent mutations are still able to cause changes in the expression of protein, its confirmation and subsequently its function (Sauna and Kimchi-Sarfaty, 2011) and many GWAS studies have revealed substantial contributions of synonymous SNPs to human disease risk and complex traits in livestock (Macaya et al., 2009; Nackley et al., 2006; Ramser et al., 2008; Ibeagha-Awemu et al., 2016). Therefore, in order not to miss out on important information that can support animal breeding plans, Ibeagha-Awemu et al. (2014) supported the notion of using both non-synonymous and synonymous mutations in animal breeding.

Significant genetic association was recorded between three missense mutations and C6:0 (rs133629324, Lys65Gln, AAA > CAA, *STAT5A* gene), C11:0 (rs210512360, Ile393Val, ATC> GTC, *SCP2* gene) and C15:0 (rs133629324, Lys65Gln, AAA > CAA, *OLR1* gene). Substituting one amino acid with another can have various phenotypic effects. Missense mutation can be conservative or non-conservative in nature. In the case of a conservative mutation, the amino acid changes to another amino acid with the same properties, either hydrophobic, hydrophilic or polar in nature, etc., meanwhile a non-conservative mutation results to an amino acid with

different chemical properties. In this study, the missense mutation rs133629324 (Lys65Gln, AAA > CAA) is a non-conservative mutation since lysine is a charged amino acid while glutamine is an uncharged amino acid. Meanwhile, the missense mutation rs210512360 (Ile393Val, ATC> GTC) is a conservative mutation as both isoleucine and valine are all non-polar amino acids. Since a missense mutation can change the structure or properties of a protein, it can therefore lead to changes in the function of the protein. Other authors have reported associations in these genes (*SCP2, OLR1, STAT5A*) with milk traits. The *OLR1* gene is responsible for the transportation of fatty acids and it also binds and damages the oxidised form of low density lipoprotein (Schennink et al., 2009). *OLR1* g.8232C>A has been shown to be associated with LCFA C18, and also with C18 and CLA indices (Schennink et al., 2009). Single nucleotide polymorphism in the *STAT5A* gene have been linked with milk production traits, like milk fat percentage in Jersey, Polish Black and White cattle and United States of America Holstein cattle (Brym et al. 2004; Flisikowski et al. 2004; Khatib et al. 2008). *SCP2* is known as a non-specific lipid transfer protein and it is associated with many functions in lipid metabolism (Li et al., 2016). Variations in *SCP2* levels or absence of *SCP2* expression have been linked with irregularities in the intracellular trafficking and metabolism of cholesterol and other lipids (Klipsic et al., 2015; Martin et al., 2015).

A mutation (rs380146565) in the 3^1 UTR within the *AGPAT9* gene was significantly associated with C4:0. SNPs in the 3^1 UTR of genes might influence the expression level of genes and therefore may affect the composition of fatty acids in milk (Schennink et al., 2009). Single nucleotide polymorphism in the 3'UTR of several genes have been reported to associate significantly with various milk fatty acids (Ibeagha-Awemu et al. 2014, 2016).

4.6 Conclusion

Seven of 36 SNPs investigated showed significant associations ($P \leq 0.05$) with C4:0, C6:0, C8:0, C11:0, C15:0 and C16:0 contents in milk. Of these, three were synonymous mutations in the *AGPAT9, CPT1A* and *STAT5A* genes, three missense mutations in *OLR1, SCP2* and *STAT5A* genes, and one mutation in the 3^1 UTR of the *AGPAT9* gene. This data could support genome-

based selection methods to optimally balance individual fatty acids in milk to meet up with consumer demands for high quality and healthy food and improve our knowledge of the pathways that are invloved in bovine *de novo* milk fat synthesis.

4.7 Conflicts of interests

The authors have no conflict of interest to declare.

4.8 Acknowledgements

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4.10 Supplementary table

Supplementary table S4.1 Genotype frequencies of single nucleotide polymorphisms in studied herds.

Gene	rs #	Location and characteristic of SNP	Genotype	count	Frequency	Allele	Frequency
UCP2	rs109407261	5[1] UTR	CC	490	0.869	C	0.933
			CT	72	0.128	T	0.067
			TT	2	0.004		
			NA's	13			
	rs378396093	5[1] UTR	AA	490	0.873	A	0.936
			AG	70	0.125	G	0.064
			GG	1	0.002		
			NA's	16			
	rs381477406	5[1] UTR	CC	2	0.004	C	0.059
			CG	63	0.112	G	0.941
			GG	500	0.885		
			NA's	12			
	rs383439735	5[1] UTR	AA	490	0.884	A	0.940
			AG	62	0.112	G	0.060
			GG	2	0.004		
			NA's	23			
INSIG1	rs110728454	3[1] UTR	CC	237	0.417	C	0.654
			CT	270	0.475	T	0.346
			TT	62	0.109		
			NA's	8			
	rs111011933	Missense (His270Arg, CAC > CGC)	AA	112	0.200	A	0.463
			AG	296	0.528	G	0.537
			GG	153	0.273		
			NA's	16			
	rs132633746	Missense (Ala276Gly, GCA > G**G**A)	CC	61	0.110	C	0.349
			CG	265	0.478	G	0.651
			GG	228	0.412		
			NA's	23			
	rs41256951	3[1] UTR	CC	222	0.405	C	0.648
			CT	266	0.485	T	0.352
			TT	60	0.109		
			NA's	29			
	rs42305262	3[1] UTR	CC	503	0.916	C	0.958
			CG	46	0.084	G	0.042
			NA's	28			

FBP2	rs132777464	Synonymous (Lys108Lys, AAG >AAA)	CC	144	0.259	C	0.512
			CT	281	0.505	T	0.488
			TT	131	0.236		
			NA's	21			
	rs133155622	Synonymous, (Ala166Ala., GCA >GCG)	AA	165	0.304	A	0.576
			AG	295	0.543	G	0.424
			GG	83	0.153		
			NA's	34			
OLR1	rs133629324	Missense (Lys65Gln, AAA > CAA)	GG	4	0.007	G	0.098
			TG	102	0.181	T	0.902
			TT	457	0.812		
			NA's	14			
STAT5B	rs133665517	Missense (Gly40Ser, GGC > AGC)	CC	454	0.809	C	0.901
			CT	103	0.184	T	0.099
			TT	4	0.007		
			NA's	16			
PIGZ	rs134024539	5[1]UTR	AA	60	0.110	A	0.353
			AT	265	0.486	T	0.647
			TT	220	0.404		
			NA's	32			
	rs382020465	5[1]UTR	CC	406	0.724	C	0.851
			CT	143	0.255	T	0.149
			TT	12	0.021		
			NA's	16			
KCNMAI	rs134429681	Synonymous (Tyr343Tyr, TAC > TAT)	AG	15	0.026	G	0.513
			GG	562	0.974	A	0.487
ACAD8	rs41784334	3[1] UTR	CC	1	0.002	C	0.043
			CG	46	0.082	G	0.957
			GG	512	0.916		
			NA's	18			
	rs41784332	3[1] UTR	CC	165	0.309	C	0.570
			CT	279	0.522	T	0.430
			TT	90	0.169		
			NA's	43			
	rs41784334	3[1] UTR	CC	1	0.002	C	0.043
			CG	46	0.082	G	0.957
			GG	512	0.916		
			NA's	18			
	rs470242182	Missense (Val90Ala, GTG > GCG)	AA	567	1.000	A	1.000
			NA's	10			
STAT5A	rs378205101	Synonymous (Pro268Pro, CCC > CCT)	AG	129	0.230	A	0.615
			GG	432	0.770	G	0.385

			NA's	16			
	rs380833204	Synonymous (Asn264Asn, AAC > AAT)	CC	429	0.793	C	0.896
			CT	112	0.207	T	0.104
			NA's	36			
	rs448701112	Synonymous (Ile285Ile, ATT > ATC)	AA	442	0.817	A	0.905
			AG	95	0.176	G	0.095
			GG	4	0.007		
			NA's	36			
	rs137182814	Synonymous (Thr329Thr, ACC > ACG)	CC	183	0.323	C	0.578
			CG	288	0.509	G	0.422
			GG	95	0.168		
			NA's	11			
DB1	rs210945785	5[1]UTR	CC	76	0.144	C	0.386
			CT	256	0.485	T	0.614
			TT	196	0.371		
			NA's	49			
SCP2	rs211310529	Missense (Ile128Val, ATT > GTT)	TT	576	1.000	T	1.000
			NA's	1			
	rs210512360	Missense, (Ile393Val, ATC> GTC)	CC	15	0.027	C	0.171
			CT	160	0.288	T	0.829
			TT	380	0.685		
			NA's	22			
AGPAT9	rs380146565	3[1] UTR	GG	338	0.610	G	0.788
			GT	197	0.356	T	0.212
			TT	19	0.034		
			NA's	23			
	rs482502090	3[1] UTR	CC	373	0.659	C	0.819
			CT	181	0.320	T	0.181
			TT	12	0.021		
			NA's	11			
	rs43167426	Synonymous (Thr162Thr, ACC > ACT)	CC	7	0.013	C	0.158
			CT	157	0.290	T	0.842
			TT	377	0.697		
			NA's	36			
PIK3CG	rs435689622	Missense (318Val >Glu, GTG > GAG)	AA	577		A	1.000
IGSF9B	rs876095260	Missense (His351Asn, CAC > AAC)	GG	577		G	1.000
	rs876383654	Missense(Gly1085Ala, GGC>GCC)	GG	577		G	1.000
TRIB3	rs210704544	5[1]UTR	CC	134	0.250	C	0.533
			CT	303	0.565	T	0.467
			TT	99	0.185		
			NA's	41			

	rs207783172	Missense (Pro60Leu, CCG > CTG)	GG	577		G	1.000
CPT1A	rs42199236	Synonymous (Asn88Asn, AAC > AAT)	CC	51	0.093	C	0.324
			CT	253	0.462	T	0.676
			TT	244	0.445		
			NA's	29			

CHAPTER 5

5.0 GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION

Generally, this study was aimed at investigating the effects of dietary supplementation of cows' diets with USFA on blood and milk component phenotypes and the regulatory mechanisms underlying these effects; as well as examine association between SNPs in genes in fatty acid biosynthesis pathway and de novo synthesized fatty acid profiles. I concentrated on the effects of USFA on milk and blood metabolites and the molecular processes involved in the response of the mammary gland to supplemental USFA. Ruminant milk, especially cow milk is an important food source for man. It is a rich source of proteins, energy, minerals (e.g. calcium) and vitamins (A, B, D, E and K) in human nutrition. Milk supplies USFA with positive effects on human health (Parodi, 2005). Unsaturated fatty acids are associated with decreased risk of cardiovascular diseases (stroke, high blood pressure, heart failure and coronary heart diseases), inflammatory diseases and some types of cancers (Kris-Etherton and Innis, 2007; Griel and Kris-Etherton, 2006; Parodi, 2005) and have protective roles against several chronic diseases and therefore, may increase human longevity (Solfrizzi et al., 2005). Saturated fatty acids, mainly C12:0, C14:0 and C16:0 are considered to negatively affect human health when consumed in excess. Saturated fatty acids comprise about 70% of the lipids in milk. To improve wellness, a reduction in the overall consumption of SFAs, trans-fatty acids and cholesterol has been put forth while emphasis has been placed on the need to increase intake of USFA (Kris-Etherton and Innis, 2007; Griel and Kris-Etherton, 2006; Salter et al., 2011). Dietary manipulations of milk fat composition have been achieved through feeding of materials rich in USFA (e.g. soybean oil, SFO, LSO, canola oil, fish oil, etc.). This strategy has shown to reduce yields of fatty acids of all chain lengths. In particular, *de novo* synthesized fatty acids are decreased to a greater extent and this result in a shift in milk fatty acid profile such that the

proportion of short and medium chain fatty acids are decreased while longer chain and USFAs are increased (Bauman and Griinari, 2001).

Few studies have examined the after effects of dietary supplementation with USFA on the physiology of the animal including blood metabolites and milk components or for how long effects are active after withdrawal of USFA supplements. I presented the results of the first part of my Ph.D. research project in chapter 2, which investigated the treatment and post-treatment effects of dietary supplementation with 5% SFO or 5% LSO on milk components and blood metabolites of Canadian Holstein cows. In this study, we demonstrated the post treatment effects of dietary USFA on milk components and blood metabolites and showed that, the residual effects of feeding USFA on the physiology of dairy cows were still active up to three weeks after cessation of treatments. We observed an increase in blood NEFA and TAG levels during treatment with USFA. Triacylglyceride forms approximately 98% of milk lipid and dietary supplementation with 5% LSO or 5% SFO probably resulted to increased availability of ruminal biohydrogenation products of PUFA (Ibeagha-Awemu et al., 2016), which were absorbed and eventually transported and stored in the adipose tissues and liver as TAG. Meanwhile, hydrolysis of TAG stored in the adipose tissues yields NEFA (free fatty acids) and glycerol.

In this study, we observed a steady increase in the concentration of BHBA throughout the experimental period. Results of studies in which blood BHBA concentrations were measured after dietary supplementation with USFA are not consistent. For example, Alizadeh et al., (2010) reported a steady decline in BHBA in cows fed diets supplemented with safflower seeds at the early stage of lactation while Mendoza et al. (2008) noted increased BHBA concentrations when diets of grazing cows were supplemented with whole sunflower seeds (rich in PUFA), which is consistent with our data. Additionally, we demonstrated the effects of dietary supplementation with USFA on milk components like somatic cell count, fat, protein, lactose and MUN. We observed the influence of supplemental feeding on MUN concentration as it declined significantly during the TP and returned to control levels exactly one week after withdrawal of treatments. Several authors did not observe significant effects of diets rich in USFA on MUN concentrations (Marchesini et al., 2009; Sigl et al., 2010). The major end product of nitrogen metabolism in dairy cows is MUN, and it is synthesized primarily in the liver and eventually transported in blood to the kidney where it is excreted in urine. It is found

in milk of dairy cows because its concentration equilibrates rapidly with other body fluids while in blood including milk (Gustafsson and Palmquist, 1993). Due to excellent farm management practices during the entire experimental periods, we observed no effect of treatments on milk SCC. However, the use of SCC to monitor possible infections is normally meant to detect "clinical mastitis", which dramatically increases SCC counts. Additionally, SCC counts are not precise enough to be sure that there is no "subclinical infections" where the bacteria are present and affect the mammary gland integrity and metabolism. Furthermore, a better monitoring of possible subclinical infection by bacteriology tests on each quarter throughout the monitoring period would be important in future studies.

Furthermore, we investigated the effects of dietary supplementation with SFO or LSO on milk fat content. Treatments reduced milk fat content by 34.2% (LSO) and 29.9% (SFO), which is consistent with other studies (Bell et al., 2006; Murphy et al., 2008; Angulo et al., 2012). Milk fat provides energy for the body and is an important determinant of the nutritional quality of milk. Milk fat composition is particularly responsive to nutrition (Lock and Bauman, 2004; Chillard et al., 2000) thus providing avenues for its manipulation for human benefit. Numerous studies have considered the effect of nutrition on milk fat synthesis in ruminants (reviews by Bauman and Griinari, 2003; Chilliard et al., 2007; Shingfield and Griinari, 2007, Shingfield et al., 2010).

A new design for future studies will include two more groups, one control group having no treatment to account for differences that can occur in time, one group fed with saturated fat to account for differences due to a supplement of "fat" in the diet. Finally, 4% supplementation with dietay USFA will be preferable since 5% have a negative influence on feed intake.

Several genes influence the physiological processes in response to nutrients in the mammary gland. Transcriptomic analysis by the method of next generation sequencing was carried out using mammary biopsy from these animals following dietary supplementation with 5% LSO or 5% SFO. Transcriptome analysis revealed 1,006 and 199 genes that were significantly differentially regulated by LSO and SFO respectively, meanwhile miRNA transcriptome analysis detected 14 and 22 miRNAs significantly differentially regulated by LSO and SFO, respectively (Ibeagha-Awemu et al., 2016; Li et al., 2015). Assessing gene expression without

taking into account the factors that regulate their activities may not adequately explain the complex biological mechanisms underlying the expression of complex traits like blood metabolites and milk components since genes do not act in isolation. Instead, genes and regulatory factors work in concert to influence the phenotypic expression of traits. The Weighted Gene Co-expression Network Analysis (WGCNA) approach was used to better understand the highly connected miRNAs (miRNA clusters or modules) and miRNA-mRNA interactions involved in the regulation of blood metabolites and milk components following dietary supplementation with USFA. Thus, the mRNA and miRNA transcriptome data (Ibeagha-Awemu et al., 2016; Li et al., 2015) was used for co-expression network analysis and three consensus modules, blue, brown and turquoise with miRNA membership of 70, 34 and 86, respectively, were identified in the bovine mammary gland following dietary supplementation with 5% LSO or 5% SFO. We correlated important miRNA modules with phenotypes (milk and blood components). Additionally, target genes (mRNA) of miRNAs from modules significantly correlated with traits were enriched to explore the possible biological processes, pathways and transcriptional regulators of phenotypes. Finally, miRNA-mRNA networks regulating the milk and blood phenotypes were identified. In this study, significant positive correlation between the blue module with lactose in SFO treatment was found. The brown module was positively correlated with C12:0, C22:0, C18:1n9c, MUFA, 10, 12-CLA and C17:0 in LSO treatment while the turquoise module was significantly correlated with several traits including protein percentage in LSO treatment and protein yield, milk yield, C14:0, C18:3n3n and 9, 11-CLA in SFO treatment. Cell cycle arrest was the GO term commonly enriched in the three modules. Enriched KEGG pathways common to the 3 modules were p53 signaling pathway and TGF-beta signaling pathway. The most significantly correlated miRNA-mRNA pairs were bta-miR-183/*RHBDD2* (P =0.003), bta-miR-484/*EIF1AD* (P =0.011) and bta-miR-130a/*SBSPON* (P =0.004) in the blue, brown and turquoise modules respectively. These results suggest that the blue, brown and turquoise modules miRNAs, hub miRNAs, miRNA-mRNA networks, cell cycle arrest GO term and common enriched KEGG pathways have considerable influence on milk and blood phenotypes following dietary supplementation of dairy cow diets with USFA.

Dietary techniques (feeding dairy cattle with diets rich in USFA) which was the first part of my Ph.D. studies, has been used by dairy producers in order to increase the amount of beneficial FA in milk (Ibeagha-Awemu et al., 2016 ; Shingfield et al., 2013). However, the effect of feeding is reversed when the feed source is withdrawn (Shingfield et al., 2013). Also, there is low efficiency of transforming dietary fatty acids into milk USFA as a result of ruminal biohydrogenation of dietary FA in the rumen of the animal. Messenger ribonucliec acid transcriptome analysis revealed 18 genes of the fatty acid and lipid biosynthesis pathways that were differentially expressed following dietary supplementation with 5 % LSO or 5 % SFO (Ibeagha-Awemu et al., 2016). In the third part of my Ph.D. studies, potential functional SNPs were mined from the RNA-Seq data of these genes and their assocaition with *de novo* synthesized milk fatty acid profiles of Canadian Holstein cows was determined. The biosynthesis of bovine milk fat is a complicated process regulated by many genes belonging to several pathways (Bauman et al., 2011; Mele et al., 2009; Mach et al., 2011). Furthermore, lipid metabolism in the mammary gland of dairy cows is highly controlled at the level of the transcriptome (McNamara, 2011). Candidate gene studies have shown that polymorphisms in key lipogenic genes have important effects on milk fat composition (Mele et al., 2007; Moioli et al., 2007; Schennink et al., 2007, 2008; Kgwatalala et al., 2009a, b, c; Conte et al., 2010). In our study, there were significant associations (P <0.05) between seven SNPs in five genes and C4:0 (rs380146565, *AGPAT9* gene), C6:0 (rs133629324, *STAT5A* gene), C8:0 (rs43167426, *AGPAT9* gene), C11:0 (rs210512360, SCP2 gene), C15:0 (rs133629324, STAT5A gene) and C16:0 (rs137182814, *STAT5A* gene, and rs42199236, *CPT1A* gene).

This identified genetic variations associated with *de novo* -synthesized fatty acid in bovine milk could facilitate breeding for decreased milk SFA.

5.2 GENERAL CONCLUSION

Dietary supplementation of cows' diets with 5% SFO or 5% LSO influenced blood metabolites and milk components during the treatment and PTP. Milk fat content decreased by 34.2% (LSO) or 29.9% (SFO) during treatments and its concentrations returned to control levels after three weeks of cessation of treatments. Milk protein content increased slightly during treatment (significant for SFO by day +28) and decreased to control levels one week after cessation of treatments. Milk urea nitrogen was decreased by both treatments and levels increased rapidly following withdrawal of treatments reaching control levels by day +35, exactly one week after cessation of treatments. Unlike NEFA, the concentration of TAG did not return to control levels even after 28 days (day+56) of withdrawal of treatment. Overall, BHBA levels continued to increase after withdrawal of treatments, reaching significant levels by day +49 (three weeks after cessation of treatment). Our data shows that the residual effects of feeding USFA on the physiology of dairy cows were still active up to three weeks after cessation of treatments.

Additionally, the WGCNA approach and mRNA/miRNA read data was used to identify modules or clusters of highly connected genes in order to understand the interaction of the major regulatory factors (highly inter-connect miRNAs and mRNA-miRNA) involved in the regulation of cow's response to dietary supplementation with USFA. In this study, three consensus miRNA modules were identified (blue, brown and turquoise) with miRNA membership of 70, 34 and 86, respectively. It was demonstrated how miRNAs in these modules interacted with mRNA to influence blood and milk phenotypes following dietary supplementation with USFA. Hub miRNAs for the blue, brown and turquoise modules were bta-miR-30d, bta-miR-484 and bta-miR-16b, respectively. Turquoise module had significant correlations with several traits (protein yield, fat yield, milk yield and fat percentage) in CP but with only protein percentage in LSO treatment and protein yield, milk yield, C14:0, C18:3n3n and 9, 11-CLA with SFO treatment. The association of miRNA modules with milk and blood phenotypes has provided information about miRNA modules, hub miRNAs, GO terms, TFs and pathways that are involved in the regulation of blood and milk parameters following dietary

supplementation with diets rich in USFA. This study will contribute to the understanding of the co-expression patterns of miRNAs, miRNA/mRNA and regulatory activities in the bovine mammary gland following dietary supplementation with USFA. Further studies are needed to validate the miRNA modules and mRNA-miRNA pairs associated with milk and blood components in this study.

In the genetic association study between *de novo* synthesized FA and SNPs, thirty-six SNPs in 18 genes were genotyped in 577 DNA samples from Canadian Holstein cattle from 18 herds in Quebec. Association analysis between genotypes and FA was carried out statistically.

Association analysis indicated that seven SNPs (rs380146565, rs133629324, rs43167426, rs210512360, rs133629324, rs42199236, rs137182814) in *AGPAT9, STAT5A, SCP2, OLR1* and *CPT1A* genes were associated significantly ($P < 0.05$) with C4:0, C6:0, C8:0, C11:0, C15:0 and C16:0 FA.

This study will assist in unravelling the genetic variations that are involved in *de novo* synthesis of bovine milk FA. Additionally, the identified associated genetic variations could facilitate breeding for decreased milk *de novo* synthesized FA. Genetic information from this study, coupled with nutritional information can be used to strategically develop management schemes to influence milk fat content and the composition of individual FA in milk fat of dairy cows

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